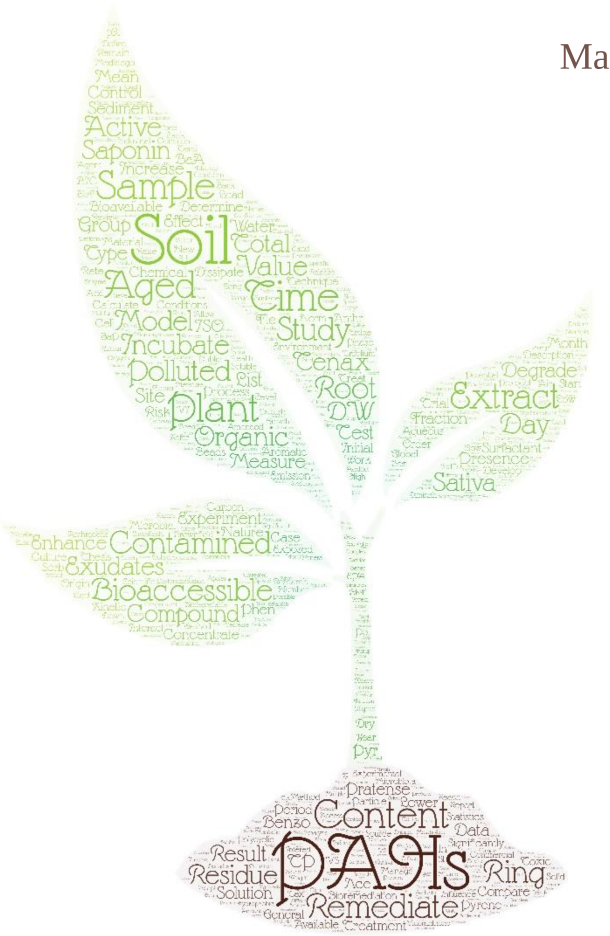
LIÈGE université
Gembloux
Agro-Bio Tech

Investigating the effects of plants and their root exudates on PAHs bioaccessibility to soil microorganisms and their impact on aged-contaminated brownfields remediation

Marie DAVIN



COMMUNAUTÉ FRANÇAISE DE BELGIQUE
UNIVERSITÉ DE LIÈGE – GEMBLOUX AGRO-BIO TECH

INVESTIGATING THE EFFECTS OF PLANTS AND THEIR ROOT EXUDATES ON PAHS BIOACCESSIBILITY TO SOIL MICROORGANISMS AND THEIR IMPACT ON AGED-CONTAMINATED BROWNFIELDS REMEDIATION.

Marie DAVIN

Dissertation originale présentée en vue de l’obtention du grade de docteur en sciences agronomiques et ingénierie biologique

Promoteurs : Prof. Gilles COLINET & Prof. Marie-Laure FAUCONNIER
Année civile : 2020

Résumé

Le lourd passé industriel de nos régions a généré un nombre considérable de friches (environ 6.000 en Région Wallonne et 3,5 millions en Europe) qui nécessitent une réhabilitation en vue de leur revalorisation. Les hydrocarbures aromatiques polycycliques (HAPs) sont des composés organiques persistants qui s'accumulent dans l'environnement et représentent environ 17% des polluants à traiter.

L'objectif de cette thèse s'inscrit dans le développement de techniques biologiques de remédiation des HAPs dans les sols de friches industrielles, en particulier la bioremédiation et la phytoremédiation. Ces techniques constituent des alternatives aux techniques classiquement utilisées dans les stratégies de dépollution des sols, souvent agressives, disruptives et onéreuses. La thèse qui s'articule autour de plusieurs axes s'est construite sur l'observation largement rapportée dans la littérature scientifique d'une diminution des teneurs en HAPs dans les sols en présence de plantes. L'hypothèse est que cette diminution serait liée à la production d'exsudats racinaires.

Un protocole de mesure de la bioaccessibilité, basé sur une extraction à l'aide de billes de Tenax®, a été adapté à deux sols contaminés issus de friches industrielles. Pour les deux sols, les cinétiques de désorption des HAPs ont été établies et décrites par des modèles continus de type « site distribution » et des temps d'extraction permettant la comparaison d'échantillons de sols ont été calculés (respectivement 48 h et 24 h pour chacun des sols).

Le potentiel d'une saponine commerciale comme agent d'extraction et comme dopant de bio-remédiation sur un sol à contamination ancienne a été étudié. Dans une première expérimentation, les HAPs ont été extraits des échantillons de sol avec des solutions de saponine (0; 1; 2; 4 et 8 g L^{-1}). Lors d'une seconde expérimentation, des échantillons de sol ont été incubés à 28°C durant 14 et 28 jours en présence de saponine (0; 2,5 and 5 mg g^{-1} MS). Les émissions de CO_2 ont été suivies tout au long de l'expérience et l'activité déshydrogénase a été mesurée après incubation afin d'évaluer l'activité microbiologique du sol. Les teneurs en HAPs résiduels et bioaccessibles ont aussi été déterminées. Comparée à l'eau, la solution de saponine à 4 g L^{-1} a extrait significativement plus de HAPs dans leur globalité. Après 28 jours, la présence de saponine n'a augmenté ni l'atténuation des HAPs ni leur bioaccessibilité par rapport à un échantillon de contrôle. Cependant, les émissions de CO_2 et les activités déshydrogénase ont été significativement plus importantes en présence de saponine, suggérant l'absence d'effet toxique des surfactants à l'encontre du microbiote du sol.

L'effet de deux types d'exsudats racinaires de *Fabaceae* (*Medicago sativa* L. ou *Trifolium pratense* L.) sur l'atténuation et la bioaccessibilité des HAPs dans un sol à contamination ancienne a aussi été étudié lors d'une incubation à 28°C. Les émissions de CO_2 ont été significativement plus importantes en présence d'exsudats de *T. pratense* ; l'activité déshydrogénase (mesurée après 14 et 28 jours) a montré une amélioration de l'activité microbienne du sol en présence des deux types d'exsudats comparé à un échantillon non traité de sol ; la teneur résiduelle en HAPs a plus

diminué dans le sol non traité qu'en présence d'exsudats de *T. pratense* ; et les exsudats de *M. sativa* ont diminué la bioaccessibilité des HAPs mais pas leurs teneurs résiduelles.

Les effets d'une culture de *Medicago sativa* L. et de *Trifolium pratense* L. sur la bioaccessibilité et l'atténuation des HAPs dans un sol à contamination ancienne ont été étudiés après trois, six et douze mois. Les résultats montrent un meilleur développement de *M. sativa* sur le sol contaminé. En l'absence de plante (contrôle) ou en présence de plantes chétives (*T. pratense*), la teneur résiduelle en HAPs dans la rhizosphère est atténuée d'environ 90% par rapport au contenu initial ; en présence de *M. sativa* l'atténuation n'a été que de 50% après douze mois. La bioaccessibilité des HAPs a augmenté plus significativement en l'absence de plante.

Enfin, une analyse critique de trois postures scientifiques qui guident les études de dépollution des sols contaminés aux HAPs a été effectuée. (i) Le choix des composés polyaromatiques étudiés et ciblés dans la littérature scientifique a été discuté en suggérant que les 16 HAPs de la liste de surveillance de l'agence environnementale de protection américaine (US-EPA) n'est plus suffisante pour rencontrer la réalité des défis environnementaux. (ii) Le choix des échantillons expérimentaux a été abordé via l'utilisation de mesures bibliométriques pour démontrer le manque d'études de remédiation utilisant des sols à contamination ancienne ou présentant des co-contaminations. (iii) L'utilisation plus systématique du protocole de mesure de la bioaccessibilité (ISO/TS 16751:2018) lors d'études de remédiation a été recommandée afin d'améliorer l'étude des processus et l'établissement de stratégies de gestion des sols.

Abstract

In Wallonia, there are 6,000 estimated brownfields (rising to over 3.5 million in Europe) that require remediation. Polycyclic aromatic hydrocarbons (PAHs) are persistent organic compounds of major concern that tend to accumulate in the environment, threatening ecosystems and health. They represent 17% of all encountered pollutants.

The thesis, articulated around several axes, focused on improving less aggressive PAHs remediation techniques in brownfields, such as bioremediation and phytoremediation, as alternatives to environmentally aggressive, expensive and often disruptive current soil remediation strategies. More specifically, it was built on several authors' observation that PAHs soil contents decrease in the presence of plants. The hypothesis was made that this could be related to the production of plant root exudates.

A bioaccessibility measurement protocol was adapted to two brownfield soils using Tenax® beads in order to compare PAHs bioaccessibility in soil samples. In both experimental soils, PAHs desorption kinetics were established, described by site distribution models, and common extraction times were calculated (respectively 48 h and 24 h for both experimental soils).

The potential of saponin (a natural surfactant) as extracting agent and as a bioremediation enhancer on an aged-contaminated soil was investigated. In a first experiment, soil samples were extracted with saponin solutions (0; 1; 2; 4 and 8 g L^{-1}). In a second experiment conducted in microcosms (28°C), soil samples were incubated for 14 or 28 days in presence of saponin (0; 2.5 and 5 mg g^{-1} DW). CO_2 emissions were monitored throughout the experiment. After the incubation, dehydrogenase activity was measured as an indicator of microbiological activity, and the bioaccessible and residual PAHs contents were determined. The 4 g L^{-1} saponin solution globally extracted significantly more PAHs than water. Neither PAHs dissipation nor bioaccessibility were enhanced in presence of saponin compared to control samples after 28 days. However, CO_2 emissions and dehydrogenase activities were significantly more important in presence of saponin, suggesting no toxic effect of this surfactant towards soil microbiota.

The role of two *Fabaceae* (*Medicago sativa* L. or *Trifolium pratense* L.) root exudates in enhancing PAHs bioaccessibility and dissipation in an aged-contaminated soil was investigated during an incubation experiment (28°C). The CO_2 emissions were significantly higher in presence of *T. pratense* exudates; the dehydrogenase activities (measured after 14 and 28 days) showed improvements of the soil microbial activity in presence of both types of root exudates compared to untreated soil samples; the PAHs residual contents decreased more in untreated samples than in the presence of *T. pratense* exudates; and *M. sativa* exudates lowered PAHs bioaccessibility but not residual contents.

The effects of *Medicago sativa* L. and *Trifolium pratense* L. on the PAHs' bioaccessibility and dissipation in an aged-contaminated soil throughout a rhizoremediation trial were investigated. The bioaccessible and residual PAH contents were quantified after three, six and twelve months of culture. The rhizoremediation

results show that *M. sativa* developed better than *T. pratense* on the contaminated soil. When plants were absent (control) or small (*T. pratense*), the global PAHs' residual contents dissipated from the rhizosphere to 8% and 10% of the total initial content, respectively; in the presence of *M. sativa*, dissipation after 12 months was only 50% of the total initial content; and the PAHs bioaccessible content increased more significantly in the absence of plants.

Finally, a review of three scientific trends that lead PAHs contaminated soils/sediments remediation studies and management was conducted. (i) The choice of PAHs compounds that are being studied and targeted in scientific literature were discussed, and it was suggested that the classical 16 PAHs from the American Environmental Protection Agency's (US-EPA) watch list might no longer be sufficient to meet actual environmental challenges. (ii) The choice of experimental material in remediation studies was discussed. Bibliometric measures were used to show the lack of PAHs remediation trials based on co-contaminated or aged-contaminated material. (iii) The systematic use of the recently validated bioaccessibility measurement protocol (ISO/TS 16751:2018) in remediation trials was discussed, and it was suggested that such measurement should be implemented as a tool to improve remediation processes and management strategies.

Remerciements

La thèse… Un long parcours de formation, de recherche, de découverte et d'apprentissage, parfois par l'erreur. Ponctué de ses moments de doute, de joie, de sentiment d'échec ou au contraire d'accomplissement, c'est un travail de longue haleine durant lequel on peut parfois se sentir bien seul. Mais le plus grand enseignement que j'en tire, c'est que rien n'aurait été possible sans soutien.

Tout d'abord, le promoteur. Ou en l'occurrence, les promoteurs. Aussi différents et complémentaires que peuvent l'être le jour et la nuit, Marie-Laure Fauconnier et Gilles Colinet ont chacun rempli ce rôle. Leurs conseils ou remarques, tantôt prodigués avec discrétion, tantôt avec fracas, ont toujours su me donner l'impulsion nécessaire pour avancer dans ce projet. Merci à vous deux, *j'ai fait pour un mieux*.

Merci aussi à mon comité de thèse, anciens et nouveaux membres : Gilles Colinet, Marie-Laure Fauconnier, Sarah Garré, Anne-Lise Hantson, Georges Lognay et Arnaud Monty. Merci de m'avoir guidée et encouragée durant cette thèse.

Merci à Jean-Marc Aldric et à Philippe Jacques d'avoir accepté d'être dans le jury de la thèse.

Cette thèse s'est déroulée sur fond d'assistanat. J'ai une pensée pour les professeurs Laurent Bock, Frédéric Boulvain, Gilles Colinet, Marie-Laure Fauconnier et Georges Lognay pour lesquels j'ai enseigné durant ces années. Chaque cours m'a apporté son lot de défis, mais surtout d'expériences inoubliables qui m'ont fait grandir et il est certain que tout ça me manquera.

Et en parlant d'enseignement, je pense aussi aux membres des équipes pédagogiques qui, au fil des années, m'ont tour à tour enseigné rigueur, souplesse ou patience : Alex, Amandine, Bérénice, Claire, Félix, Laurie, Manon, Maurine, Michaël, Madame Nathalie, Stéphanie, Pilou, sans oublier Béatrice, Danny, Franck, Raphaël, Thomas et Vincent. Merci à vous pour ces riches moments.

Merci aussi aux étudiants. Ceux à qui j'ai enseigné, avec qui je suis partie en excursion, mais surtout ceux avec lesquels j'ai eu le plaisir de travailler sur la thèse : Airy, Amandine, Emilie, Bastien et Elisa, rien n'aurait été possible sans votre aide précieuse !

Ma thèse s'est déroulée au sein de deux services, eux aussi bien différents et uniques en leur genre, où j'ai eu la chance d'être accueillie à bras ouverts. Il serait tentant de les mentionner séparément, mais « la CGO » et « la pédo » ont toutes deux joué leur rôle et m'ont fait évoluer durant ces années.

Merci à Christophe pour ses Cent Wafers, à Kévin pour ses pamplemousses soigneusement épluchés à l'Opinel, à Billo pour ses cornets de pâtes, à Gaëtan pour ses tartes professionnelles, à Tierry pour ses poulets « PDG » et à Thomas pour ses Alpro soja chocolat et ses sandwichs interminables.

Merci à Florent et ses râleries qui animent les pauses, à Tierry et son goût des singes, à Pierre-Yves et Saskia pour les parties de carte, à Chloé et son cross-fit, à Christophe et Danny pour leurs séances « secrets d'histoire de Gembloux » et à tous les autres : Amaury, Aurore, Brieuc, Catherine, Clémence, Clément, Damien, Emilie L, Emilie M, Evelyne, Fatimata, Jean-Charles, Jean-Thomas, Jonas, Laurie, Malorie, Martin, Mathilde, Matthew, Maxence, Oriane, Pauline, Pierre-Patrick, Serge, Sophie, Victor, Xavier. Tous, et ceux que j'oublie sûrement, vous avez su créer des ambiances variées et riches qui font vivre une équipe. Maintenant que nous sommes obligés de moins nous côtoyer, je me rends compte que j'ai eu raison d'en profiter !

Merci à ceux qui ont partagé mon quotidien d'un peu plus près et ont su tantôt créer une ambiance de bureau studieuse, tantôt aider à relâcher la pression. Kacem et ses téléphones, Saskia et nos parties de rangement de labo, Félix et sa concentration à toute épreuve couplée à une distraction exemplaire !

Et puis il y a les mercis spéciaux. A Kévin pour m'avoir enseigné les rudiments du codage. A Danny et Thomas pour les séances de sauvetage de l'HPLC. A Franck, présence tranquille et apaisante, expert en bébé, achat et rénovation de maisons. A Sébastien pour nos séances concierge et notre série *j'allais juste aux toilettes*. A Françoise pour nos séances d'archéologie dans les armoires. Enfin surtout, à Amandine, qui a su me distiller conseils, remarques, bons plans, confidences et fou-rires. Sans tes séances coaching *à fond les ballons* c'est sûr, ça aurait été plus laborieux.

Il y a aussi le soutien qui semble venir de plus loin mais n'en est pas moins réel. Anne-Sophie, Bernard, Diana, Emilie, Sophie, Sophie et Damien, Damien et Lorraine, Bart et Renée, vous étiez probablement plus convaincus que moi que j'allais en voir le bout, de cette thèse. Merci pour votre intérêt, votre soutien et vos encouragements.

Merci aux copines de Saint-Jacques. Auré, Juju, Lala, vous êtes de cette famille qu'on se choisit pour avancer, grandir, se construire. Entre nous, plus vraiment besoin de mots. Merci pour votre soutien indéfectible.

Il m'est impossible d'exprimer toute ma reconnaissance et ma gratitude envers vous Papa, Maman et Pierre. Soutiens inconditionnels, fans de l'ombre, vous me portez depuis toujours, et rien ne me paraît impossible grâce à vous. Mention spéciale à toi, Papa, relecteur infatigable et confident imperturbable. *Tu sais bien hein…*

Seb, merci à la vie de t'avoir mis sur ma route, deux fois, et merci à toi d'y être resté. C'est sûr, tu as eu le rôle le plus ingrat. Merci pour nos débats, parfois animés mais toujours rigoureux, pour ta patience, ta compréhension, tes conseils et tes mouchoirs. Merci de partager ma vie.

Enfin, merci à cette flamme qui grandit au fond de moi et qui a su me donner l'impulsion pour cette dernière ligne droite. Tout est, comme toujours, question de motivation.

Table of contents

List of figures

List of tables

List of supplementary tables

List of abbreviations

∑2-3 rings	Sum of light PAHs of three rings or less (N, Ace, Fle, Phen and Anthr),
∑4 rings	Sum of intermediate PAHs of four rings (F and Pyr)
∑4-6 rings	Sum of heavy PAHs of four rings or more (BaA; Chrys, BbF, BkF, BaP, DBahA, BghiP and IcdP)
∑all	Sum of all PAHs (N to IcdP)
Ace	Acenaphtene
Anthr	Anthracene
BaA	Benzo[a]anthracene
BaP	Benzo[a]pyrene
BbF	Benzo[b]fluoranthene
BghiP	Benzo[ghi]perylene
BIC	Bayesian information criterion
BkF	Benzo[k]fluoranthene
BTEXs	Group of monoaromatic compounds comprising benzene, toluene, ethylbenzene, xylenes, and styrene
C	Unplanted control samples
$C_{ext\ t}$	Amount of PAH extracted by Tenax® beads after t hours of contact [µg g^{-1} DW]
CD	Cyclodextrins
Chrys	Chrysene
CMC	Critical Micellar Concentration
$C_{tot\ in}$	Total initial PAH concentration in the soil [µg g^{-1} DW]
DBahA	Dibenzo[ah)anthracene
DMSO	Dimethylsulfoxide
DOM	Dissolved organic matter
DW	Dry Weight
E_MS	*Medicago sativa* L. root exudates
E_TP	*Trifolium pratense* L. root exudates
F	Fluoranthene
Fle	Fluorene
GC-MS	Gaz chromatography coupled to mass spectrometry detection
HOCs	Hydrophobic organic compounds
HPCD	Hydroxypropyl-β-cyclodextrin
HPLC-FLD	High performance liquid chromatography coupled to fluorescence detection
HRGC-MS/MS	High resolution gas chromatography coupled to ion trap and negative chemical ionisation
IARC	International Agency for Research on Cancer
IcdP	Indeno[123-c,d]pyrene
INTF	Iodonitrotetrazolium formazan

k_H	Henry’s constant
log K_{OW}	Octanol-water partition coefficient
log K_{OC}	Water-organic carbon partition coefficient
MRM	Multiple reaction monitoring mode
MS	*Medicago sativa* L. or samples planted with *Medicago sativa* L.
MW	Molecular weight
N	Naphtalene
NAPLs	Non-aqueous phase liquids
NER	Non-extractable residue
NRC	American National Research Council
p°	Vapour tension
PACs	Polycyclic aromatic compounds
PAHs	Polycyclic aromatic hydrocarbons
PCBs	Poly-chloro biphenyls
PCDDs	Poly-chloro-dibenzo-p-dioxines
PCDFs	Poly-chloro-dibenzo-furanes
PGPR	Plant growth promoting rhizobacteria
Phen	Phenanthrene
PM	Particulate matter
POPs	Persistent organic pollutants
Pyr	Pyrene
s	Aqueous solubility
S_0	Initial sorbed fraction
SDS	Sodium dodecyl sulfate
SOM	Soil organic matter
S_t	Sorbed fraction of compound remaining after t hours of extraction
t_{ex}	Extraction time (for bioaccessibility measurement)
TP	*Trifolium pratense* L. or samples planted with *Trifolium pratense* L.
US EPA	American Environmental Protection Agency
VI	Intervention value (of the 2008 Walloon legislation): value over which soils are to be cleaned-up
VR	Reference value (of the 2008 Walloon legislation): ideal value to reach for the remediation of historical pollutions, and mandatory value to reach for the remediation of a new pollution in the 2008 legislation
VS	Threshold value (of the 2008 and 2018 Walloon legislation): value above which a risk-assessment (in case of a historical pollution), and a mandatory clean-up (in case of a new pollution) are to be implemented in both legislations
WFD	Water Framework Directive
WHO	World Health Organization

Introduction

Main concepts and hypothesis

Intense industrial activity has been the sign of economic and societal thrive since the industrial revolution. It has allowed increases in people's income and health, as well as the rapid acquisition of many technologies and knowledge. However, and after more than two centuries, human development and thriving have come to face new challenges and now need to be redefined in a sustainable way. The United Nations have established 17 goals that are to be achieved through sustainable development, which is a delicate balance and interaction between three elements: social inclusion, economic growth, and environmental protection (UN, 2020). The present work settles in this third pillar: environmental protection. Indeed, and for a long time, environmental repercussions of human actions have not been a priority, until a global awakening started to take place around the 1970's. At first, people realised that careless use and disposal of resources could have an impact on their health, for instance if their immediate environment was polluted or damaged. This came along with the realisation that environmental resources are not infinite, are not always renewable, and that their reckless use and disposal can have serious influences on global health, not only humans' (Keith, 2015).

When it comes to the environment, and though it should be considered as a whole, it is more common to approach the matter through its main components: the hydrosphere, the lithosphere, the atmosphere, and the biosphere. And because humans tend to have a very significant impact on the other spheres, scientists have come to consider a fifth sphere, the anthroposphere, apart from the biosphere. Of course, all the spheres interact and it would be both reductive and incorrect to study one of them without considering the others. For example soil, which will be at the centre of this thesis, is defined as being at the interface of all these spheres as it is made of about 50% of solid particles (mineral and organic), of 50% of pores filled with water and/or air, and as it supports the development and life of macro- and micro- organisms (Brady and Weil, 2008).

Soil is a sink for water, air and nutrients that are essential to life, but also for contaminants or even pollutants. As a reminder, a contamination is defined as *"the presence of elevated concentrations of substances in the environment above the natural background level [...]"*, whilst a pollution is a contamination resulting in deleterious effects (FAO, 2020). This is precisely what this work focusses on. For years, industrial activities have produced and released contaminants in the environment. Either by diffuse emissions in the atmosphere, which have spread on long distances around the emission source before being deposited on soil, or by local spills on or in the ground, leading to more concentrated but also more localised plumes of contamination. Brownfields represent a big fraction of the various types of land that might have accumulated pollution. Such lands have generally been at the centre of intense industrial activity in the past and their future reuse is compromised by the (potential) presence of hazardous substances (EPA, 2020). In the USA, there is an estimated 450,000 brownfields (EPA, 2020). In Europe (28 countries), there are 650,000 registered sites where polluting activities have taken or are still taking place (JRC, 2018). In Wallonia (Belgium), there are 17,400 potentially contaminated sites, that represents an average of 1-10 sites / 10 km² (RW, 2018). An inventory is still in progress and so far, 2,213 sites (for a total of 37.95 km²) have been identified as in need for some rehabilitation. Whether these sites are polluted or not sometimes remains to be determined (RW, 2020). Such numbers outline the ubiquity of potentially degraded land, and more importantly of pollution hazards. Besides, brownfields represent huge potential economic losses, as they often present advantages such as good geographic configuration and situation, but are unused because of potential or effective pollution. They are also an important health hazard, since it is likely the pollution they contain has been left unmanaged for years, potentially damaging the environment. Because many brownfields yet have to be managed, and often remediated, this thesis focusses on the remediation of brownfield polluted soil, as it is representative of actual sustainability and management challenges.

Polluting compounds are of various nature, and their introduction in the environment might have been unintentional, for example if they are produced as a process' by-product or if they are accidentally spilled. Classically, pollutants are described as either inorganic or organic. Metal elements such as cadmium, lead or copper, constitute the majority of a well-defined list of inorganic pollutants. On the other hand, organic contaminants present a much larger variety of compounds. The most encountered groups of organic pollutants are aliphatic hydrocarbons, mono-aromatic hydrocarbons, such as benzene, toluene, ethylbenzene, xylenes, and styrene (BTEXs) but also phenols, halogenated hydrocarbons (usually classified as organic solvents), polycyclic aromatic hydrocarbons (PAHs), poly-chloro-biphenyls (PCBs), poly-chloro-dibenzo-p-dioxines (PCDDs), or poly-chloro-dibenzo-furanes (PCDFs). The previous list is not exhaustive and will soon have to include new members known as emerging pollutants. Indeed recent research has started to highlight the potential hazard of antibiotics, hormones, and pharmaceutical molecules that have been detected in the environment (Reichert *et al.,* 2019). Organic pollutants thus present a very wide range of groups and have to account for new members rather regularly. Besides, each group is made of many different compounds. For example, PCBs, PCDDs and PCDFs are chemical families respectively made of about 209, 75 and 135 congeners. Of course, not all those compounds are stereochemically stable, some are very rare, and some are more toxic than others. For instance, twelve PCBs are really toxic, seven PCBs represent about 80% of global occurrences, and only one of them is common to both subgroups (Lemière *et al.,* 2008). This shows that it would be both incredibly difficult and probably useless to consider all existing organic compounds when discussing organic pollution remediation. Thus, choices have been made regarding the list of priority pollutants, and it is sometimes very interesting to discover why some pollutants made it to a watch list over others. But such discussion will be for later (Part 4). For now, let us introduce PAHs, the group of organic pollutants that have been the focus of this thesis, and more specifically which PAH compounds were dealt with.

1. What are PAHs?

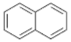
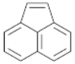
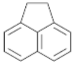
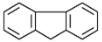
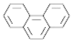
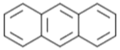
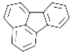
Polycyclic aromatic hydrocarbons are classically defined as hydrophobic organic compounds made of two or more condensed aromatic rings. The rings can be arranged in linear, angular or clustered shapes, leading to a very large variety of compounds (Ghosal *et al.,* 2016). These compounds can be of natural or anthropogenic origin. Their largest natural origin is petrogenic since PAHs are formed in petroleum products due to a series of diagenetic processes. They are also formed in living organisms due to biogenic processes, and are commonly formed during incomplete combustions (forest fires, volcanic eruption…), in which case they are of pyrolytic origin. Anthropogenic sources of PAHs into the environment are mostly incomplete combustion (waste incineration, car exhaustion, industrial activity…) and accidental spilling (Iqbal *et al.,* 2008; Dhar *et al.,* 2019). It is interesting to note that though commonly presented as undesired combustion by-products, there are several industrial applications for PAHs as they are implied in the fabrication of pharmaceutical products, lubricants, dyes, resins… (Abdel-shafy & Mansour, 2016)

Because of the name of this group of molecules, it is common to assume they are only composed of carbon and hydrogen. And whilst it is possible to encounter compounds with alkyl substitutions, it is also important to mention that many compounds exist that are either substituted with oxy or hydroxyl functions, halogens, or can even contain sulphur or oxygen atoms within some of their cycles, which makes them heterocycles (Idowu *et al.,* 2019). Such structural variety enlarges the PAHs family to the group of polycyclic aromatic compounds (PACs), which will be discussed again later (Part 4). For now, let us simply specify that the compounds of interest at the centre of the presented research are strictly PAHs, as they originally are only made of carbon and hydrogen, and are classically referred to as the 16 US-EPA priority pollutants. Indeed, these compounds have been on a “Priority Pollutant” watch list established by the American Environmental Protection Agency (US-EPA) for over four decades, and at the centre of most research conducted by the scientific community. Besides, and from a more practical point-of-view, those 16 PAHs are also part of soil regulations in Belgium, which is where the thesis was conducted. And because the thesis wanted to bring an applicable dimension to its research, the Belgian soil regulations provided a concrete basis on which to lean.

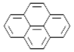
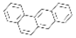
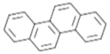
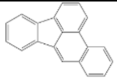
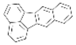
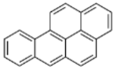
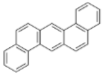
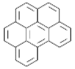
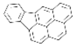
The matter that still needs to be addressed is why those compounds are listed as priority pollutants. Because of their structure, PAHs tend to become rapidly hydrophobic, lowly volatile, and also poorly soluble in water as the number of condensed rings increases (Table 1). Several physicochemical parameters are used to describe and predict PAHs fate in the environment. Aqueous solubility (s) and vapour tension (p°) show that PAHs have a general low tendency to migrate in the aqueous and gaseous compartments of the environment, but it is only a general observation. Henry’s constant (k_H, the ratio between the vapour tension and the aqueous solubility of a compound), describes more thoroughly whether a compound can diffuse in one or both these compartments. Regarding the 16 PAHs exposed in Table 1, most of them will have a tendency to migrate in the environment through the aqueous phase, although this tendency is weak given their overall low solubility. Two other important indicators are the octanol-water partition coefficient (log K_{OW}) and the water-organic carbon partition coefficient (log K_{OC}). Both sets of values show that PAHs in general will partition more into hydrophobic compartments or onto carbon-containing particles, and more so as log K_{OW} and log K_{OC} increase. This means that PAHs will be accumulated along the food chain, and will also tend to sorb onto soil particles (Lemière *et al.*, 2008). Such phenomenon can occur directly when PAHs are spilled onto soil, but also as they are emitted in the atmosphere, in which case they will sorb to particulate matter and be deposited through dry or wet processes, sometimes far from their emission point (Abdel-shafy & Mansour, 2016). In soil, other factors such as salinity, temperature or the presence of dissolved organic matter influence sorption. Ultimately, PAHs tend to physically migrate into condensed organic matter and inaccessible micropores from the soil. This sequestration phenomenon implies a decline in PAHs availability and is called ageing (Mahanty *et al.*, 2011). It will be discussed again later. However, it does not mean that PAHs remain immobile in the environment, as they can migrate to surface or ground water through their particulate form. It is this tendency to accumulate into organisms and in the environment that makes PAHs part of the persistent organic pollutants (POPs).

Organisms' exposure to PAHs can occur though inhalation (e.g. smoke or particulate matter), ingestion (water, soil or food) or dermal contact (e.g. with contaminated soil). Toxic effects are highly variable depending on the dose of exposition but also on the PAHs mixture, as several of them are usually present together. In case of acute exposition, symptoms such as nausea or irritation can occur whilst a chronic exposition can lead to lung, kidney or liver abnormalities. Finally, it is the metabolism of PAHs that can lead to carcinogenic effects. As will be explained later, PAHs are metabolized into phenols, quinones, diols, epoxides… which are highly reactive and can bind to genetic material or cellular proteins, leading to abnormal gene expression, inheritable genetic mutations, and tumours (Moorthy *et al.*, 2015). Therefore, PAHs are classified by the International Agency for Research on Cancer (IARC) as potential or effective carcinogens to humans (Table 1), which is why they have been studied as priority pollutants in many environmental remediation studies.

Table 1. Structural, physicochemical and toxicological properties of the 16 studied PAHs compounds. INERIS physicochemical groups: LMW are low molecular weight PAHs of 2-3 rings, IMW are intermediate molecular weight PAHs of 4 rings, and HMW are high molecular weight PAHs of 4 rings or more. IARC toxicity groups: 1 is carcinogenic to humans, 2A is probably carcinogenic to humans, 2B is possibly carcinogenic to humans, 3 is not classifiable as to its carcinogenicity to humans, and 4 is probably not carcinogenic to humans (IARC, 2020; INERIS, 2020).

Compound	CAS number	Structure	Molecular Formula	Number of cycles	Molecular Weight **M** (g mol^{-1})	Aqueous Solubility **s** (mg L^{-1}) at 25°C	Vapour Tension **p°** (Pa) at 25°C	[a] Henry's constant $\mathbf{k_H}$ (Pa m^3 mol^{-1}) at 25°C	Octanol-Water Partition Coefficient $\mathbf{log\ K_{OW}}$	Water-Organic Carbon Partition Coefficient $\mathbf{log\ K_{OC}}$	INERIS physico-chemical group	IARC toxicity group
Naphthalene	91-20-3		$C_{10}H_8$	2	128.2	32	10.5	42.1	3.3	3.15	LMW	2B
Acenaphthylene	208-96-8		$C_{12}H_8$	3	152.2	3.9	0.89	34.7	4.07	1.4	LMW	NA[b]
Acenaphtene	83-32-9		$C_{12}H_{10}$	3	154.2	3.7	0.36	15.0	3.92	3.66	LMW	3
Fluorene	86-73-7		$C_{13}H_{10}$	3	166.2	1.9	0.09	7.87	4.18	3.89	LMW	3
Phenanthrene	85-01-8		$C_{14}H_{10}$	3	178.2	1.2	0.09	13.4	4.57	3.16	LMW	3
Anthracene	120-12-7		$C_{14}H_{10}$	3	178.2	1.29	0.00036	$4.97\ 10^{-2}$	4.45	4.41	LMW	3
Fluoranthene	206-44-0		$C_{16}H_{10}$	4	202.3	0.26	0.0012	0.93	5.1	4.86	IMW	3

(continued)

Compound	CAS number	Structure	Molecular Formula	Number of cycles	Molecular Weight **M** (g mol^{-1})	Aqueous Solubility **s** (mg L^{-1}) at 25°C	Vapour Tension **p°** (Pa) at 25°C	[a] Henry's constant **k_H** (Pa m^3 mol^{-1}) at 25°C	Octanol-Water Partition Coefficient **log K_{OW}**	Water-Organic Carbon Partition Coefficient **log K_{OC}**	INERIS physico-chemical group	IARC toxicity group
Pyrene	129-00-0		$C_{16}H_{10}$	4	202.3	0.13	0.012	18.7	5.32	4.86	IMW	3
Benzo [a]anthracene	56-55-3		$C_{18}H_{12}$	4	228.3	0.0057	$2.6\ 10^{-5}$	1.04	5.61	5.25	HMW	2B
Chrysene	218-01-9		$C_{18}H_{12}$	4	228.3	0.002	$8.4\ 10^{-5}$	9.59	5.16	5.12	HMW	2B
Benzo [b]fluoranthene	205-99-2		$C_{20}H_{12}$	5	252.3	0.0012	$6.7\ 10^{-5}$	14.1	6.57	5.18	HMW	2B
Benzo [k]fluoranthene	207-08-9		$C_{20}H_{12}$	5	252.3	0.0008	$1.3\ 10^{-8}$	$4.10\ 10^{-3}$	6.84	5.90	HMW	2B
Benzo[a]pyrene	50-32-8		$C_{20}H_{12}$	5	252.3	0.0038	$7.3\ 10^{-7}$	$4.85\ 10^{-2}$	6.06	6.74	HMW	1
Dibenzo [ah]anthracene	53-70-3		$C_{22}H_{14}$	5	278.3	0.0005	$1.3\ 10^{-8}$	$7.24\ 10^{-3}$	6.70	6.15	HMW	2A
Benzo [ghi]perylene	191-24-2		$C_{22}H_{12}$	6	276.3	$2.6\ 10^{-4}$	$1.3\ 10^{-8}$	$1.38\ 10^{-2}$	6.5	4.98	HMW	3
Indeno [1,2,3-cd]pyrene	193-39-5		$C_{22}H_{12}$	6	276.3	0.062	$1.3\ 10^{-8}$	$5.79\ 10^{-5}$	6.18	6.80	HMW	2B

[a] k_H values were calculated based on **s** and **p°** values: $k_H = p°/s$.

[b] NA = data not available

2. How to remediate PAHs in soil?

First and foremost, it is important to make an essential distinction between inorganic and organic pollutants. Inorganic pollutants being mostly metallic elements, they are non-degradable. Therefore, remediation treatments will always result in the displacement, harvest, or immobilization of the inorganic pollutants, whereas many organic pollutants are degradable, either through biological or chemical processes involving combustion or oxidation. This implies that if a treatment is well conducted and optimized, an organic pollutant may actually disappear. On the down side, a poorly conducted degradation may also lead to undesired metabolites that very well might be more toxic than the original pollutant, as will be exposed later.

Soil remediation techniques have been studied and developed for decades. They are classically described as treatments *in situ* or *ex situ*, meaning with the soil still in place or excavated, and “on site” or “off site”, meaning the soil is either being treated on its original location or it is being moved, treated, or even disposed of, to another location (Colombano *et al.*, 2010). These seemingly simple differences have implications on the possible extent, cost, and disruptive aspects of the remediation. Indeed, a site that is being excavated will have to be refilled later, either with the treated soil or another one, and this will have important influences on soil structure, stability and compaction due to several physical manipulations (e.g. sieving), but also on future biological functions if toxic chemicals are used. On the other hand, some sites present such concentrated and deep plumes of pollution that existing technologies would be too expensive, or not efficient enough, to treat them. In such cases the reasonable economic choice could be to excavate the soil and confine it, either on site between impermeable geomembranes (such technique is called encapsulation) or into industrial landfills. This way of managing pollution, though it does not truly diminish it, is sometimes the best way of managing the site. Because land management and soil remediation both take place in an economic frame, the best compromise must be found between rendering value to a site by lowering its pollution to accepted guidelines, whilst not giving it too much added value by using exorbitant remediation techniques.

Regarding techniques themselves, they tend to be presented in the literature as being either physical, chemical, or biological even though the distinction is often pretty tenuous between a category and another. A brief overview of techniques employed to manage PAHs polluted soil will be made but as there are many guidelines and reviews describing remediation techniques and their applicability to different types of contaminants, they will not be extensively developed (Table 2).

Table 2. Classification of a few techniques employed in PAHs soil remediation.

Treatments		***Ex situ***		***In situ***
Localisation		**On site**	**Off site**	**On site**
Preliminary manipulations				
Excavation		X	X	
Crushing		X	X	
Particle screening		X	X	
Transport			X	
Physical/ thermal techniques				
Stabilisation		X	X	X
Desorption		X	X	X
Incineration		X	X	
Pyrolysis		X	X	
Vitrification		X	X	X
Venting				X
Electroremediation				X
Encapsulation		X		
Landfill Disposal			X	
Chemical techniques				
Washing				
	Water	X	X	X
	Organic solvents	X	X	X
	Vegetable oil	X	X	X
	Surfactants (synthetic / natural)	X	X	X
	Complexing agents	X	X	X
Oxidation				
	Ozone	X	X	X
	Fenton’s reagent	X	X	X
	Permenganate, …	X	X	X
	Photocatalytic degradation (UV lights)	X	X	X

(continued)

Treatments		***Ex situ***		***In situ***
Localisation		**On site**	**Off site**	**On site**
Biological techniques				
Bioremediation				
	Bioslurry	X	X	
	Biopile	X	X	
	Composting	X	X	
	Bioventing			X
Phytoremediation				
	Phytoextraction / phytoaccumulation			X
	Phytostabilization			X
	Phytotransformation / phytodegradation			X
	Phytovolatilization			X
	Rhizodegradation / phytostimulation			X
	Rhizofiltration / phytopumping			X

Physical treatments aim the displacement, the immobilization and sometimes the degradation of the pollution from the soil that is being treated. *Ex situ* techniques start with excavation, where the polluted area is removed. Then, the soil can undergo several treatments. Crushing and particle screening will concentrate the pollution in the thin size fraction. This fraction can then be washed or simply disposed of in industrial landfills. Of course, the washing effluents (water sometimes amended with chemical reagents) will have to be treated later on. Another possibility is to excavate and encapsulate polluted soil on site in order to prevent and control the release of pollution into the environment through leaching or washout. Leachates coming from the capsule have to be monitored and treated as well. Soil can also be mixed with stabilizing agents (e.g. concrete) to prevent pollution migration or undergo thermal treatment to desorb or even burn organic contaminants. Depending on the used temperature and the presence or absence of oxygen, the process is either named desorption, incineration, or pyrolysis (Colombano *et al.*, 2010). Concerning *in situ* techniques, the use of heat can go from thermal desorption, which will release pollutants in the air, to vitrification, which will melt the soil and all its components to immobilize any pollutant. Venting, or soil vapour extraction, is the forced circulation of air through the soil still in place in order to extract contaminants in a gaseous phase. This can also be coupled to heating. Of course, the outcoming air phase will have to be treated. Finally, electrokinetic treatment uses a low-voltage current that goes through soil to mobilize and accumulate the pollutants towards the electrodes (Sakshi *et al.,* 2019). Soil washing, which was mentioned earlier, is a much-diversified type of treatment. Though it is classically considered a physical treatment, it can also be classified as a chemical treatment, especially because it is sometimes considered as being part of the "solvent extraction" treatments. The simplest soil washing technique only uses water. But because of the low aqueous solubility and affinity of hydrophobic compounds for water, several techniques have been developed to try and enhance organic pollutants extraction from the soil. The use of organic solvents (e.g. ethanol, acetone or dichloromethane), vegetable oil (e.g. sunflower or peanut), surfactants (of synthetic or natural origin) or complexing agents (e.g. cyclodextrins) are the most studied (Gan *et al.,* 2009; Von Lau *et al.,* 2014).

The investigation of surfactants has been at the centre of many remediation publications, not only in soil washing technologies. Indeed, and as will be developed later on, the fact that surfactants can potentially increase the apparent aqueous solubility of hydrophobic compounds is also being investigated in the area of bioremediation as bioavailability enhancers. Surfactants can be synthetic or natural; cationic, anionic, or non-ionic compounds. In general, non-ionic surfactants are less toxic and provide a better solubilisation enhancement than the ionic ones. Because of synthetic surfactants overall low biodegradability and toxicity, searchers have been focussing their attention on the use of more renewable, degradable, and thus eco-friendly, natural surfactants. Such "biosurfactants" can be microbial-based compounds (e.g. glycolipids, lipopeptides, or phospholipids), or plant-based compounds (e.g. saponin), even though the latter have generally been less investigated (Mulligan *et al.,* 2001; Lamichhane *et al.,* 2017). It is important to keep in mind that all previously mentioned washing and extracting techniques could easily become very expensive because of the use of chemical reagents, energy, but also because outcoming effluents, leachates or air have to be treated if they export contaminants from the soil.

Apart from the washing technologies previously exposed, chemical treatments can also aim at the degradation of the contaminant. Chemical oxidation uses oxidants such as ozone, permanganate, Fenton's reagent (Fe(II)-H_2O_2)… for the remediation of recalcitrant organic compounds. The objective is to partially or completely degrade the initial contaminant into more degradable metabolites or carbon dioxide and water. Although less common, photocatalytic degradation is also a chemical oxidation process that relies on hydroxyl radicals to degrade the organic pollutant. The radicals are created by the exposition of oxidants such as ozone to UV lights (Colombano *et al.,* 2010; Mahanty *et al.,* 2011; Sakshi *et al.,* 2019). The use of such strong oxidants, though showing promising results, is also more difficult to control. Besides, the use of such broad-spectrum compounds on a living soil goes along with heating and acidification phenomena (Ranc, 2017) which would have consequences on the future functions of the soil, and not only on the diminution of organic pollution.

Biological treatments are the use of biological systems, such as living bacteria, fungi, algae or plants, or their products to remediate a pollution. Several outcomes such as the immobilization, degradation or even complete mineralization are possible (Mahanty *et al.,* 2011).

Microbial degradation, or biodegradation, pathways have been intensively studied for PAHs. An overview of the state of knowledge regarding microbial catabolism pathways will be given but extensive details will not be presented since many reviews have been documenting knowledge and progress over the years (Cerniglia, 1992; Ghosal *et al.,* 2016; Nzila, 2018; Peng *et al.,* 2018; Dhar *et al.,* 2019). It is mainly the PAHs aerobic degradation by bacteria that has been studied, even though fungi are capable of degrading them too (Ghosal *et al.,* 2016). Also, PAHs degradation mechanisms in anaerobic conditions have been more studied lately (Nzila, 2018; Dhar *et al.,* 2019). Briefly, anaerobic catabolism is based on the use of iron (III), nitrate, sulfate, or manganese (IV) as electron acceptors instead of dioxygen, which will result in the formation of iron (II), molecular nitrogen, hydrosulfide or manganese (II), respectively. Such processes have both been observed in natural environment and studied in the laboratory and concern facultative aerobic bacteria (e.g. from the genera *Hydrogenophaga*, *Microbacteria*, or *Pseudomonas*) or strict anaerobic bacteria (e.g. *Delta-proteobacteria*) (Dhar *et al.,* 2019).

Aerobic catabolism requires oxygen, as it will serve as final electron acceptor, and as substrate for the hydroxylation of an aromatic ring. Procaryotic microorganisms first hydroxylate the aromatic ring using dioxygenase enzymes (and to a lesser extent monooxygenase enzymes), forming *cis*-dihydrodiols. Then the *cis*-dihydrodiol compounds are rearomatized by dehydrogenases to form diol intermediates until they are cleaved into intermediates (e.g. catechol) by intradiol or extradiol ring cleaving dioxygenases. These intermediates then enter the regular metabolic pathway (Krebs' cycle) (Cerniglia, 1992; Ghosal *et al.,* 2016). Bacteria that were frequently identified as capable of PAHs degradation are members of the genera *Pseudomonas*, *Sphingomonas*, *Mycobacteria*, *Rhodococcus*, *Acinetobacter*, *Klebsiella*, *Flavobacteria*…(Cerniglia, 1992; Dhar *et al.,* 2019).

Eucaryotic microorganisms such as fungi mostly rely on co-metabolism to degrade PAHs, as they cannot use PAHs as a primary source of energy. Two major pathways exist, depending on the (non)-ligninolytic nature of the fungi. The non-ligninolytic pathway is actually similar to the metabolic pathway encountered in mammals and involves monooxygenase enzymes from the cytochrome P450. Briefly, this cytochrome enzymes form unstable arene oxides through ring epoxidation, then a dihydroxylation transforms the unstable oxide into a *trans*-dihydrodiol. It is also possible for the unstable arene epoxide to be rearranged into phenols through non-enzymatic pathways. It is important to keep in mind that all previously mentioned epoxides and dihydrodiols are carcinogens and are thus potentially more toxic than their original parent PAHs. Non-ligninolytic fungi that were identified as capable of PAHs degradation are members of the genera *Penicillium*, *Aspergillus*, *Cunninghamella*, *Fusarium*…(Dhar *et al.,* 2019).

On the other hand, ligninolytic fungi, though they are capable of similar metabolic pathways as non-ligninolytic fungi, also produce ligninolytic enzymes. Such enzymes are either lignin peroxidases, manganese peroxidases, or phenol oxidases (laccases) that will create hydroxyl free radicals. These radicals will oxidize PAHs into quinones and acids, which are much less carcinogenic than dihydriols. And because they are extracellular enzymes, they have the ability to reach immobilized PAHs through diffusion and are not being secreted due to the presence of PAHs (Cerniglia, 1992; Ghosal *et al.,* 2016). Ligninolytic fungi that were identified as capable of PAHs degradation are for example *Phanerochaete chrysosporium* P. Karst, *Trametes versicolor* Lloyd, *Pleurotus ostreatus* (Jacq. ex Fr.) P. Kumm…(Dhar *et al.,* 2019).

It is important to emphasize that PAHs recalcitrance to complete mineralization or even degradation increases with the number of rings. Indeed, the more complex the molecule, the more likely metabolic pathways will lead to potentially carcinogenic dead-ends (Cerniglia, 1992). Quite often, a microbial species is not capable of completely mineralizing a PAH compound, nor even of starting its degradation in the case of heavier PAHs. However, cometabolism phenomena can take place when more than one PAH compound is present. In such scenario, a heavier and more recalcitrant compound that cannot serve as a carbon source to a microbial species will be degraded along with lighter or more readily degradable compounds (Mahanty *et al.,* 2011). Besides, it has been observed that PAHs present more extensive degradation when varied microbial consortia are present, which is the case in natural environments. It is assumed that some metabolites, though they could be considered dead-ends for some species, are metabolic intermediates to others. For example, fungi are generally not capable of completely mineralizing heavier PAHs, whilst bacteria are not capable of initiating their degradation. But ligninolytic fungi, with their extracellular enzymes, can transform heavier PAHs into smaller and more polar intermediates that can be metabolized by bacteria (Ghosal *et al.,* 2016). It would be reductive however to assume that a microbial consortium could always mineralize a PAH compound. Indeed, in a polluted natural environment, both the microorganisms and the PAHs are present in mixtures. And besides cometabolism, phenomena of augmentation or inhibition can influence both the extent or the rate of individual PAHs degradation, depending on the type of mixture but also the degrading microbial consortia (Mahanty *et al.,* 2011). Inhibition, when the presence of a compound reduces the degradation of another, is the most common effect noted. This could either be caused by competition between compounds for common enzymatic degradation pathways (Stringfellow & Aitken, 1995) or by one compound repressing the synthesis of enzymes that degrade the other (Bouchez *et al.,* 1995). On the other hand, augmentation, the enhanced degradation of a compound in presence of another, may be caused by a positive analogue effect on enzyme induction (Bouchez *et al.,* 1995). It is important to keep in mind that such phenomena were highlighted by studies conducted in controlled conditions of two or three PAHS, and though it is safe to assume that mixed and varied microbial consortia will present complementary pathways and facilitate a more extended degradation compared to single species cultures, those enhancing or inhibiting effects are not systematically encountered and are difficult to predict (Mahanty *et al.,* 2011).

The use of biodegradation to remediate pollution is named bioremediation and has been implemented in several techniques. E*x situ* techniques are for example bioslurry, biopile or composting. In bioslurry, fine soil particles are brought to suspension in water and mixed with other amendments in a reactor to stimulate degradation. Amendments can be nutrients, acids or bases to control pH… The biopile technique is similar but soils are placed under a geomembrane in order to control humidity and pollutant volatilisation. Soils are also amended with nutrients and air is blown from under the pile to provide oxygen. Composting is similar to the biopile technique except soils are not placed under a geomembrane and their oxygenation is assured by regular mixing. In all cases, leachates or water effluents have to be collected and treated. An example of *in situ* technique is bioventing, where oxygen is injected into the soil to stimulate aerobic degradation. It is similar to venting where desorption is privileged over degradation. Again, the outcoming air must be controlled for pollutants and treated afterwards (Colombano *et al.,* 2010).

Besides microorganisms, plants are also being used and studied to remediate soil pollution. There are different types of phytoremediation strategies depending on the targeted contaminant, the outcome and the mechanisms at work. Brief definitions will be given as there is sometimes some confusion regarding the vocabulary. Phytoextraction, or phytoaccumulation, will remove a contaminant from the soil, and because the contaminant is not degraded, it will accumulate in some part of the living plant. In the literature, such technique is mostly encountered for the remediation of inorganics. Phytostabilization is caused by the effect of plant roots on their environment. Root exudates can modify pH or moisture content, causing for example metals to precipitate. The outcomes are minimized mobility and interaction with the biota of the contaminants in soil. It is, again, most encountered in the remediation of inorganics. Phytotransformation, or phytodegradation, will lead to a transformation, or a degradation, of a contaminant. It takes place inside the plant, meaning the contaminant as to be soluble enough to be absorbed. Phytovolatilization results in the removal of a contaminant from the soil to the atmosphere, sometimes by converting it into a volatile form. It has been observed both on light volatile organic compounds and on inorganics such as mercury (Susarla *et al.,* 2002; Pilon-Smits, 2005). Rhizodegradation, also referred to as phytostimulation, is caused by the close interactions existing between vascular plants and the soil microbiota in their rhizosphere (Susarla *et al.,* 2002). It has been acknowledged for a long time that these living organisms have mutually beneficial interactions. For example, plant growth promoting rhizobacteria (PGPR) can positively influence plants health through the induction of systemic resistance (Jha & Saraf, 2015). On the other hand, plants can enhance microbial growth in their rhizosphere through the exudation of sugars, amino acids, or secondary metabolites (Singer *et al.,* 2003), but also by providing aeration to the soil through roots growth. Such close interactions lead to enhanced growth and microbial activity in the rhizosphere, which is suspected to enable organic contaminants biodegradation. It is important to emphasize that even though rhizodegradation is commonly considered a phytoremediation technique, it is the close interaction of plants with the soil microbiota that enables biodegradation, clearly suggesting that rhizodegradation is also a bioremediation technique. Finally, it is noteworthy to mention phytopumping, or rhizofiltration, which can be used to remove contaminants from the environment, or at least reduce their migration. Because of the transpiration process, plants are capable of pumping large volumes of water, which could lead either to the accumulation of contaminants in the root area, or even to phytoextraction (Susarla *et al.*, 2002; Pilon-Smits, 2005; Colombano *et al.*, 2010).

The use of living organisms such as plants or the soil microbiota presents advantages in the context of soil remediation like the fact that it is more eco-friendly and less destructive of the soil, especially if it is implemented *in situ*. However, such techniques are time-consuming compared to the above-mentioned physico-chemical techniques. They also might be limited by the extent, depth and variety of the pollution, which is why research is still necessary to understand and try to optimize biological remediation techniques.

3. PAHs : The bioavailability matter

PAHs biodegradation is influenced by many environmental factors other than the composition of the microbial consortia that could metabolise the compounds. First, factors such as pH, salinity, soil moisture, temperature, water-dissolved oxygen, or mineral nutrients will provide conditions more or less favourable to the microbial activity. But no matter how efficient microbial catabolic pathways are, biodegradation processes are also a balance between the uptake and metabolism of a compound by a living cell and the mass transfer of the compound to the microbial cell (Haritash & Kaushik, 2009). Contact between a pollutant and a microbial cell mostly takes place in the soil aqueous solution (Johnsen *et al.,* 2005). But because of their physico-chemical properties (low aqueous solubility, high hydrophobicity, and molecular structure), PAHs are prone to ageing phenomena, which will reduce their presence in the soil aqueous solution throughout time. Ageing occurs when environmental components contribute to the chemical or physical segregation of compounds, thus influencing their accessibility to degrading agents or their enzymes (Masciandaro *et al.,* 2013). Ageing is driven by two main mechanisms: sorption and diffusion (also named sequestration). Both phenomena take place onto or into organic and mineral matter. Hydrogen bonds and van der Waals forces occur initially and instantly upon contact between the contaminant and the soil matter, leading to a large fraction of contaminants being sorbed, but still remaining removable. With time, sorption and intraparticle diffusion become stronger and deeper because more permanent covalent bonds will form between the contaminant and soil components of similar nature, slowly decreasing the “degradable or removable” fraction, which will successively evolve into a “readily available” fraction, a “recalcitrant” fraction, and eventually a “non-extractable” fraction (Reid *et al.,* 2000; Semple et al. 2003). It is noteworthy to precise that the non-extractable residue (NER), or fraction, will even resist strong solvent extraction methods used to determine what is classically named the “total content”. Therefore the “total PAHs concentrations” that have been measured according to norm ISO 13877 throughout the experiments presented in this thesis are technically the sum of all above-mentioned fractions, except the non-extractable fraction and thus represent the “total extractable PAHs concentrations” (Ortega-Calvo *et al.,* 2015).

Sorption and sequestration phenomena will thus decrease the availability of hydrophobic contaminants, preventing biodegradation. So in order to improve bioremediation technologies, many researches have focussed on bioavailability and its different areas.

Firstly, the description and the modelling of sorption, and also of its opposite phenomenon, desorption. Some searchers have tended to simplify the above-mentioned fractions to two fractions: one that can rapidly desorb, and one that can slowly desorb, leading to the generalised use of a first-order two-compartment desorption model throughout the literature (Barnier *et al.,* 2014). Continuous models, which assume that the contaminant fractions are distributed through a continuum of soil compartments, have been much less used even though they seem to be a better fit to the described phenomena (Connaughton *et al.*, 1993). This will be discussed in Part 2 and Part 3.

Secondly, many studies have been conducted on ways to enhance desorption in order to increase the pollutants availability to the degrading microbiota, as well as on some chemical ways to assess this bioavailability. But because of the complexity of the mechanisms, and the variety of the stages of availability contaminants can be at, many discussions on bioavailability processes and definitions have been published and reviewed in the literature (Ehlers & Luthy, 2003; Semple *et al.*, 2004; Reichenberg & Mayer, 2006; Ortega-Calvo *et al.*, 2015). A few concepts will be developed hereafter.

The generic used term is “bioavailability”, but it is defined differently depending on scientific disciplines. Therefore in 2002, the American National Research Council (NRC) settled a few definitions concerning this term in soils and sediments. Instead of defining the term, the NRC defined the “bioavailability processes” as *“the individual physical, chemical, and biological interactions that determine the exposure of organisms to chemicals associated with soils and sediments”* (Ehlers & Luthy, 2003). The definition details the different steps that will lead a contaminant from being bound to a soil particle to being absorbed by an organism (Figure 1). The different processes are (A) the release of a contaminant from being bound to a solid particle, (B) the transport of the released contaminant to an organism, (C) the uptake of a bound contaminant by an organism, and (D) the uptake of the contaminant across an organism’s physiological membrane. The subsequent incorporation of the contaminant into the living system (E) is not considered as being part of the bioavailability processes because since the contaminant has been released, the soil or sediment is not implied in this process. Also, it is important to emphasize that processes A to D can take place inside an organism if the contaminant bound to a particle is ingested or inhaled (Ehlers & Luthy, 2003). Later on, and because the NRC definitions reported by Ehlers & Luthy, (2003) lacked proper definition of the bioavailability concept, Semple *et al.*, (2004) offered such a definition by leaning on the previously described bioavailability processes. They defined the “bioavailable compound” as *“that which is freely available to cross an organism’s cellular membrane from the medium the organism inhabits at a given time.”* This definition refers to process (D) in Figure 1. The authors also offered a second definition that would refer to processes A-D in Figure 1. They defined the “bioaccessible” compound” as *“that which is available to cross an organism’s cellular membrane from the environment, if the organism has access to the compound. However, the compound may be physically removed from the organism or only available after a period of time.”* This definition clearly suggests that a compound that is bound to a soil particle and not readily available at a given moment can become available later if it is released into the organism’s medium. This term thus englobes time and desorption mechanisms as being susceptible to render contaminants available to an organism. These definitions also imply that the bioavailable or bioaccessible fractions of a contaminant pool are species-dependant, and thus that it would be reductive to assimilate microorganisms' bioavailability to humans' bioavailability, for example.

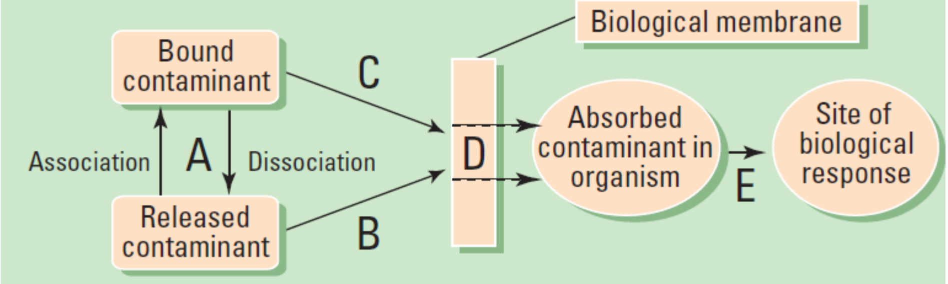


Figure 1. Bioavailability processes that lead a contaminant from being bound to a soil particle to being absorbed by an organism (in Ehlers & Luthy, (2003)). (A) is the release of a contaminant from being bound to a solid particle, (B) is the transport of the released contaminant to an organism, (C) is the uptake of a bound contaminant by an organism, (D) is the uptake of the contaminant across an organism's physiological membrane, and (E) is the incorporation of the contaminant into the living system, which is no longer considered a bioavailability process.

Along discussions on how to define bioavailability, many techniques were developed, and reviewed, that attempted to measure this bioavailability (Cui *et al.*, 2013; Cachada *et al.*, 2014). Briefly, all explored methods rely on non-exhaustive extraction of hydrophobic compounds from the soils or sediments and can be subdivided into two main categories. On one hand, chemical bioavailability measurement protocols based on mild solvents (e.g. methanol or propanol), resins (e.g. Tenax® or XAD) or complexing agents (e.g. cyclodextrins) are also known as biomimetic methods because their principle is to mimic the contaminants uptake into organisms. On the other hand bioavailability protocols based on passive samplers made of fibers, polyethylene, polymethylene and other semi-permeable materials are known as equilibrium samplers. Though seemingly close, both principles rely on two fundamentally different concepts of bioavailability that were explained thoroughly by Reichenberg & Mayer (2006) as a complement to the publications by Ehlers & Luthy, (2003) and Semple *et al.*, (2004). The authors make a distinction between "accessibility" which *"describes the mass quantity of a chemical that is or can become available within a given time span and under given conditions"* and "chemical activity" which *"quantifies the energetic state of a chemical that determines the potential for spontaneous physicochemical processes, such as diffusion and partitioning."* Accessibility is bioaccessibility defined by Semple *et al.*, (2004) and can be measured by biomimetic methods whilst chemical activity can be measured by equilibrium methods. This difference is fundamental because accessibility is operationally driven, meaning it is dependent of the nature of the targeted contaminant, of the organism, of the sample's nature, and on the measuring method's extraction time and conditions (Reichenberg & Mayer, 2006; Cui *et al.*, 2013). To put it in terms of environmental processes, bioavailability in a context of biodegradation is better defined by bioaccessibility whilst bioavailability in a context of toxicity that happens through passive diffusion is best described by chemical activity (Cui *et al.,* 2013). Thus, it is the definition and concept of bioaccessibility that was chosen in the context of this thesis. A similar choice was made in the official norm ISO/TS 16751 when it was published in 2018 and settled the debate on the determination of the bioavailable fraction of non-polar organic compounds in soil. Even though the term "bioavailability" is employed throughout the norm, its definition is clearly based on the bioaccessibility concept by Semple *et al.,* (2004).

Whilst important advances were being made on bioavailability definition and measurement, research was conducted to enhance biodegradation rates through the use of bioavailability or solubility enhancers, as previously mentioned. Principal technologies are based on the use of solvents (either water miscible or water immiscible), complexing agents or surfactants.

The use of solvents in improving PAHs biodegradation has been studied in two different ways. The use of water-miscible solvent was investigated as a way to raise PAHs solubility in the aqueous phase, similarly as surfactants are expected to act. However these solvents could either be toxic to the degrading cells or be used as a substrate in place of the pollutants, which lowers the targeted pollutant's biodegradation efficiency (Mahanty *et al.,* 2011). The use of water-immiscible solvents was investigated as a way to partition PAHs between the solvent and the aqueous phase and to control the pollutants microbial uptake. The solvent is supposed to act as a reservoir, and as cells degrade PAHs in the aqueous phase, the disequilibrium it creates causes PAHs to migrate from the solvent to the water (Daugulis, 2001). This technique theoretically enhances bioavailability and the pollutants mass-transfer rate towards the cells whilst limiting PAHs toxicity towards the degrading cells because it controls the compounds delivery to the cells. But tested solvents often ended up sequestrating the PAHs as the compounds exhibited great affinity for the immiscible solvent, therefore reducing bioavailability instead of enhancing it (Mahanty *et al.,* 2011). Besides, the immiscible solvents have to be non-biodegradable and yet biocompatible. Therefore, attempts were made to replace the immiscible solvents with solid polymers as means of controlling the pollutants delivery to the degrading cells.

Cyclodextrins (CD) were investigated as solubilisation enhancers because they could form inclusion complexes with PAHs (Cuypers *et al.,* 2002). However when partitioned into cyclodextrin complexes, PAHs were less extensively degraded (Ramsay *et al.,* 2005). But in the meantime, studies demonstrated the ability for hydroxypropyl-β-cyclodextrin (HPCD) to perform non-exhaustive extraction of hydrocarbon compounds and focused on the development of methods to evaluate the bioavailable fractions of hydrocarbons in contaminated soils (Doick *et al.,* 2005). It is one of the previously mentioned biomimetic methods that is now described in the bioavailability assessment norm ISO/TS 16751.

Surfactants were investigated to desorb hydrophobic compounds from soil into micelles (or pseudosolubilize) in order to increase their accessibility to microorganisms. As for desorption, synthetic surfactants (e.g. Triton X-100, Brij 35...) have shown promising results. But when it comes to biodegradation, negative effects were sometimes observed. Synthetic surfactants can be directly toxic to degrading organisms or indirectly as they increase toxic compounds concentrations in the environment (Mahanty *et al.,* 2011). They also may decrease the pollutants degradation by being used as a primarily carbon source by the microbiota or by sequestrating the pollutants away for the degraders (Volkering *et al.,* 1995). Therefore, research started to focus more on biosurfactants as less toxic, more degradable bioavailability enhancers. Similarly, as in washing technologies exposed earlier, the most studied surfactants are of bacterial origin and less of plant origin. As for synthetic surfactants, contrasted results have been reported where the biosurfactants either increased or decreased pollutants degradation, this by serving as carbon source or by inducing direct or indirect toxicity towards the microorganisms (Mahanty *et al.,* 2011).

4. Working hypothesis

As exposed throughout this introduction, the thesis focussed on the remediation of PAHs in brownfield polluted soils, which are also named aged-contaminated soils because brownfields have often accumulated contaminations for years or decades. Many of the existing remediation techniques are expensive, can be harmful towards the environment or can jeopardize the future use of the treated soil. Unfortunately, they are largely employed because time is of the essence as soon as an investment is being made in a brownfield remediation. However, not all brownfields present important economic interest nor are they considered worth spending millions on, which does not mean they should not be remediated or cannot be repurposed to host commercial or residential activities instead of industrial ones. Many sites like those exist, where time is not an issue but money might be. Hence, it is a great opportunity to take advantage of this available time to develop eco-friendly, and often slower, biological remediation alternatives. And since biological treatments are generally cheaper, they are also best suited for brownfields that are less of a priority.

Throughout the literature, biological remediation techniques have been explored, but mainly on artificially or freshly contaminated soils. However, they need to be brought to the next level by being tested on aged-contaminated, more representative soil in order to bring comprehension to mechanisms at work, and to be optimized. This particular thesis has been developed on the observation, reported by several searchers, that PAHs contents lower in soil in presence of vascular plants. And because of the known interactions that exist between plants and microorganisms in the rhizosphere, the bioavailability matter that often slows biodegradation mechanisms, and the fact that many plants produce and exudate surfactants in their environment, the hypothesis was formulated that some plants, through the release of their root exudates, could act as bioremediation enhancers.

Two vascular plants were at the centre of the experimentations: *Medicago sativa* L. (or alfalfa or lucerne) and *Trifolium pratense* L. (or red clover). The tested plants were chosen because they were reported by Vincken *et al.*, (2007) to synthesize saponins (natural surfactants) in their roots, but not only. The plants were also selected because the thesis wanted to lean on a realistic use in the context of Belgian brownfields remediation, and because of several advantages that meet the eco-friendly and cheap dimensions of the work. (i) They are indigenous to Belgium. (ii) They are species of the *Fabaceae* family, meaning they are nitrogen-independent. Soils from brownfields that are polluted with organic compounds tend to have very high carbon to nitrogen ratios that are not suitable for many plants culture. Thus, being nitrogen-independent constitutes an advantage to grow on such hostile soils. (iii) They are plants with hairy roots systems, which is a physical advantage in terms of reaching pollution throughout the soil. Finally, *Medicago sativa* L. has been studied previously and its potential for phytoremediation was highlighted.

In order to investigate the exposed hypothesis, the design of a few experimental setups and the implementation of analytical protocols were necessary. Also, the main hypothesis was developed into several research axes.

On the one hand, the effect of plant root exudates on PAHs bioaccessibility to soil microorganisms was studied. The hypothesis that some plant root exudates can influence PAHs bioaccessibility, and thus enhance bioremediation whilst being non-toxic towards the soil microbiota, was approached through several experiments.

First, *Medicago sativa* L. and *Trifolium pratense* L. cultures were implemented under hydroponic conditions and their respective root exudates were harvested. In the meantime, commercial saponin from *Quillaja saponaria* Molina bark, a natural surfactant, was purchased and used both in a soil extraction (or washing) experiment and in a soil incubation experiment. In the soil extraction experiment (further developed in Part 1), aged-contaminated soil was washed with aqueous solutions of saponin in order to assess whether the surfactant could enhance the apparent aqueous solubility of PAHs. In the soil incubation experiment, in microcosms (further developed in Part 1 and Part 2), aged-contaminated soil was amended with commercial saponin and incubated in controlled conditions to assess whether the surfactant could enhance PAHs bioaccessibility and dissipation, in the soil. During the incubation experiment, the potential toxicity of this amendment towards the soil microbiota was assessed through the monitoring of carbon dioxide emissions and the measurement of dehydrogenase activity, and the PAHs bioaccessibility was assessed using an extraction protocol based on Tenax® beads. Concerning the bioaccessibility measurement, when the experiments were conducted the scientific community had agreed that biomimetic methods were more appropriate to assess bioaccessibility, and scientists were about to settle on two main techniques to evaluate this bioaccessibility: the use of Tenax® beads, and the use of cyclodextrins (ISO/TS 16751:2018). But since an official norm had not yet been published, some protocol implementation was done to measure PAHs bioaccessibility in the experiments of this thesis. This is further developed in Part 2.

After the harvest of plant root exudates from both tested plants, the exudates were used in an incubation experiment, in microcosms: aged-contaminated soil was amended with raw exudates and incubated in controlled conditions to assess whether the exudates could enhance PAHs bioaccessibility and dissipation, in the soil. Bioaccessibility was also assessed using an adapted Tenax® method, and the potential toxicity of these amended exudates towards the soil microbiota was assessed through the monitoring of carbon dioxide emissions and the measurement of dehydrogenase activity as well.

The experimental results concerning the commercial saponin are exposed in Part 1, and the results concerning the raw plant root exudates are exposed in Part 2.

On the other hand, plants-PAHs interaction was studied in more realistic conditions through a rhizoremediation experiment (further developed in Part 3): aged-contaminated soil was planted with either *Medicago sativa* L. or *Trifolium pratense* L. in outdoors conditions to assess whether plants and their real exudation rates could influence PAHs bioaccessibility and dissipation.

As they were collected, most results were analysed and discussed in different papers (which have been published) and they will be discussed again in the general discussion, conclusions and perspectives of this thesis. But prior to that, a few reflections were made concerning research on PAHs remediation in aged-contaminated soil. Indeed, this thesis has been a learning process during which hypotheses and methodological choices were made. And the discussion of the results, along with the reading of the literature, have led to consider a few scientific orientations that this area of research has taken, as well as orientations research should consider leaning towards. More specifically, three topics were discussed in a critical review (Part 4) as an outcome of the experience gathered during the thesis: (i) the choice of PAHs compounds that are being studied and targeted in scientific literature, (ii) the choice of experimental material in remediation studies (freshly contaminated or aged-contaminated soil), and (iii) the need to implement a more systematic use of bioavailability in remediation trials.

5. References

Abdel-shafy, H. I., & Mansour, M. S. M., 2016. A review on polycyclic aromatic hydrocarbons: Source, environmental impact, effect on human health and remediation. *Egypt. J. Pet.*, 25(1), 107–123.

Barnier C, Ouvrard S, Robin C, Morel JL. 2014. Desorption kinetics of PAHs from aged industrial soils for availability assessment. *Sci Total Environ.* 470–471, 639–645.

Bouchez M., Blanchet D. & Vandecasteele J-P., 1995. Degradation of polycyclic aromatic hydrocarbons by pure strains and by defined strain associations: inhibition phenomena and cometabolism. *Appl. Microbiol. Biotechnol.*, 43, 156-164.

Brady and Weil, 2008. The nature and properties of soils- 14th edition, eds: Brady and Weil.

Cachada, A., Pereira, R., da Silva, E. F., & Duarte, A. C. (2014). The prediction of PAHs bioavailability in soils using chemical methods: State of the art and future challenges. *Sci. Total Environ.*, 472, 463-480. https://doi.org/10.1016/j.scitotenv.2013.11.038

Cerniglia, C. E., 1992. Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation*, 3, 351–368. https://doi.org/10.1007/BF00129093

Colombano S., Saada A., Guerin V., Bataillard P, Bellenfant G., Beranger S., Hube D., Zornig C, Giradeau I. Quelles techniques pour quels traitements°? Analyse coûts-bénéfices – BRGM/RP – 58609 – FR. Juin 2010.

Connaughton DF, Stedinger JR, Lion L.W., & Shuler M.L. 1993. Description of time-varying desorption kinetics: release of naphthalene from contaminated soils. *Environ. Sci. Technol.* 27(12), 2397–403.

Cui, X., Mayer, P., & Gan, J., 2013. Methods to assess bioavailability of hydrophobic organic contaminants: Principles, operations, and limitations. *Environ. Pollut.*, 172, 223–234. https://doi.org/10.1016/j.envpol.2012.09.013

Cuypers, C., Pancras, T., Grotenhuis, T., & Rulkens, W., 2002. The estimation of PAH bioavailability in contaminated sediments using hydroxypropyl-beta-cyclodextrin and Triton X-100 extraction techniques. *Chemosphere*, 46(8), 1235–1245. https://doi.org/10.1016/S0045-6535(01)00199-0

Daugulis, A. J., 2001. Two-phase partitioning bioreactors: A new technology platform for destroying xenobiotics. *Trends Biotechnol.*, 19(11), 457–462. https://doi.org/10.1016/S0167-7799(01)01789-9

Dhar K; Subashchandrabose SR; Venkateswarlu K; Krishnan K; Megharaj M. Anaerobic Microbial Degradation of Polycyclic Aromatic Hydrocarbons: A Comprehensive Review. In: de Voogt P (ed.) Reviews of Environmental Contamination and Toxicology. Springer Nature, Switzerland. https://doi.org/10.1007/398_2019_29

Doick, K. J., Dew, N. M., & Semple, K. T., 2005. Linking catabolism to cyclodextrin extractability: Determination of the microbial availability of PAHs in soil. *Environ. Sci. Technol.*, 39(22), 8858–8864. https://doi.org/10.1021/es0507463

Ehlers LJ, & Luthy RG., 2003. Contaminant bioavailability in improving risk assessment and remediation rests on better understanding bioavailability. *Environ Sci Technol.*, 37:295–302.

EPA, 2020. https://www.epa.gov/brownfields/overview-epas-brownfields-program (consulted May 14th 2020).

FAO, 2020. http://www.fao.org/3/x5624e/x5624e04.htm#TopOfPage (consulted May 14th, 2020).

Gan, S., Lau, E. V., & Ng, H. K., 2009. Remediation of soils contaminated with polycyclic aromatic hydrocarbons (PAHs). *J. Hazard. Mater.*, 172(2–3), 532–549. https://doi.org/10.1016/j.jhazmat.2009.07.118

Ghosal, D., Ghosh, S., Dutta, T. K., & Ahn, Y., 2016. Current state of knowledge in microbial degradation of polycyclic aromatic hydrocarbons (PAHs): A review. *Frontiers in Microbiology*, 7(1369). https://doi.org/10.3389/fmicb.2016.01369

Haritash A.K., & Kaushik C.P., 2009. Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review. *J. Hazard. Mater.* 169(1–3):1–15.

IARC, 2020 https://monographs.iarc.fr/list-of-classifications ((consulted May 14th, 2020)

INERIS, 2020. https://substances.ineris.fr/fr/page/21#fictox (consulted May 14th, 2020)

Idowu, O., Semple, K. T., Ramadass, K., O’Connor, W., Hansbro, P., & Thavamani, P., 2019. Beyond the obvious: Environmental health implications of polar polycyclic aromatic hydrocarbons. *Environ. Int.*, 123, 543–557. https://doi.org/10.1016/j.envint.2018.12.051

Iqbal, J., Overton, E.B., & Gisclair, D., 2008. Polycyclic Aromatic Hydrocarbons in Louisiana Rivers and Coastal Environments: Source Fingerprinting and Forensic Analysis. *Environ. Forensics*, 9:63-74. https://doi.org/10.1080/15275920801888301

Jha, C. K., & Saraf, M. (2015). Plant growth promoting Rhizobacteria (PGPR): a review. *J. Agric. Res. Dev.*, 5(2), 108–119. https://doi.org/10.13140/RG.2.1.5171.2164

Johnsen A., Wick L. & Harms H., 2005. Principles of microbial PAH-degradation in soil. *Environ. Pollut.*, 133(1), 71-84.

JRC, 2018. https://ec.europa.eu/jrc/en/publication/annual-reports/jrc-annual-report-2018 (consulted May 14th, 2020).

Keith, L. H., 2015. The Source of U.S. EPA's Sixteen PAH Priority Pollutants. *Polycycl. Aromat. Comp.*, 35(2–4), 147–160. https://doi.org/10.1080/10406638.2014.892886

Lamichhane, S., Bal, K. C., & Sarukkalige, R., 2017. Surfactant-enhanced remediation of polycyclic aromatic hydrocarbons: A review. *J. Environ. Manage.,* https://doi.org/10.1016/j.jenvman.2017.05.037

Lemière B., Seguin J.J., Le Guern C., Guyonnet D., Baranger Ph., Saada A., avec la collaboration de Darmendrail D., Conil P., Bodénan F., Fauconnier D., HubéD., Colombano S. – Guide sur le comportement des polluants dans les sols et les nappes – Document du BRGM 300 – Nouvelle édition 2008.

Mahanty, B., Pakshirajan, K., & Dasu, V. V., 2011. Understanding the complexity and strategic evolution in PAH remediation research. *Crit. Rev. Environ. Sci. Technol.*, 41(19), 1697–1746. https://doi.org/10.1080/10643389.2010.481586

Masciandaro G., Macci C., Peruzzi E., Ceccanti B., & Doni S., 2013. Organic matter-microorganism-plant in soil bioremediation: A synergic approach. *Rev. Environ. Sci. Biotechnol*., 12, 399-419.

Moorthy, B., Chu, C., & Carlin, D. J., 2015. Polycyclic Aromatic Hydrocarbons : From Metabolism to Lung Cancer. *Toxicol. Sci.*, 145(1), 5–15. https://doi.org/10.1093/toxsci/kfv040

Mulligan, C. N., Yong, R. N., & Gibbs, B. F., 2001. Surfactant-enhanced remediation of contaminated soil: A review. *Eng. Geol.*, 60(1–4), 371–380. https://doi.org/10.1016/S0013-7952(00)00117-4

Nzila, A., 2018. Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons under anaerobic conditions: Overview of studies, proposed pathways and future perspectives. *Environ. Pollut.*, 239, 788–802. https://doi.org/10.1016/j.envpol.2018.04.074

Ortega-Calvo, J. J., Harmsen, J., Parsons, J. R., Semple, K. T., Aitken, M. D., Ajao, C., Eadsforth, C., Galay-Burgos, M., Naidu, R., Oliver, R., Peijnenburg, W. J. G. M., Römbke, J., Streck, G., & Versonnen, B., 2015. From Bioavailability Science to Regulation of Organic Chemicals. *Environ. Sci. Technol.*, 49(17), 10255–10264. https://doi.org/10.1021/acs.est.5b02412

Peng, X., Xu, P. F., Du, H., Tang, Y., Meng, Y., Yuan, L., & Sheng, L. P., 2018. Degradation of polycyclic aromatic hydrocarbons: A review. *Appl. Ecol. Environ. Res.*, 16(5), 6419–6440. https://doi.org/10.15666/aeer/1605_64196440

Pilon-Smits, E., 2005. Phytoremediation. *Annu. Rev. Plant Biol.*, 56, 15–39. https://doi.org/10.1146/annurev.arplant.56.032604.144214

Ramsay, J. A., Robertson, K., VanLoon, G., Acay, N., & Ramsay, B. A., 2005. Enhancement of PAH biomineralization rates by cyclodextrins under Fe(III)-reducing conditions. *Chemosphere*, 61(5), 733–740. https://doi.org/10.1016/j.chemosphere.2005.03.060

Ranc B., 2017. Oxydation chimique *in situ* de la zone non saturée de sols contaminés par du goudron de houille : du laboratoire au terrain. PhD thesis : Université de Lorraine.

Reichenberg, F., & Mayer, P., 2006. Two complementary sides of bioavailability: accesibility and chemical activity of organic contaminants in sediments and soils. *Environ. Toxicol. Chem.*, 25(5), 1239-1245. https://doi.org/10.1897/05-458R.1

Reichert, G., Hilgert, S., Fuchs, S., & Azevedo, J. C. R., 2019. Emerging contaminants and antibiotic resistance in the different environmental matrices of Latin America. *Environ. Pollut.*, 255(113140). https://doi.org/10.1016/j.envpol.2019.113140

Reid, B. J., Jones, K. C., & Semple, K. T., 2000. Bioavailability of persistent organic pollutants in soils and sediments - a perspective on mechanisms, consequences and assessment. *Environ. Pollut.*, 108(1), 103-112. https://doi.org/10.1016/S0269-7491(99)00206-7

RW, 2018. http://etat.environnement.wallonie.be/contents/indicatorsheets/SOLS%205.html (consulted May 14th, 2020).

RW, 2020. http://lampspw.wallonie.be/dgo4/site_sar/index.php/presentation/index (consulted August 18th, 2020).

Sakshi, S. K., & Haritash, A. K., 2019. Polycyclic aromatic hydrocarbons: soil pollution and remediation. *Int. J. Environ. Sci. Technol.*, 16, 6489–6512. https://doi.org/10.1007/s13762-019-02414-3

Semple KT, Morriss AWJ, Paton GI., 2003. Bioavailability of hydrophobic organic contaminants in soils: fundamental concepts and techniques for analysis. *Eur. J. Soil. Sci.*, 54, 809–818.

Semple KT, Doick KJ, Jones KC, Burauel P, Craven A, Harms H., 2004. Peer Reviewed: Defining Bioavailability and Bioaccessibility of Contaminated Soil and Sediment is Complicated. *Environ. Sci. Technol.*, 38(12), 228A-231A.

Singer, A. C., Crowley, D. E., & Thompson, I. P., 2003. Secondary plant metabolites in phytoremediation and biotransformation. *Trends Biotechnol.*, 21(3), 123–130. https://doi.org/10.1016/S0167-7799(02)00041-0

Stringfellow, W. T., & Aitken, M. D., 1995. Competitive metabolism of naphthalene, methylnaphthalenes, and fluorene by phenanthrene-degrading pseudomonads. *Appl. Environ. Microbiol.*, 61(1), 357–362. https://doi.org/10.1128/aem.61.1.357-362.1995

Susarla, S., Medina, V. F., & McCutcheon, S. C., 2002. Phytoremediation: An ecological solution to organic chemical contamination. *Ecol. Eng.*, 18(5), 647–658. https://doi.org/10.1016/S0925-8574(02)00026-5

UN, 2020. https://www.un.org/sustainabledevelopment/development-agenda/ (consulted May 11th, 2020)

Vincken JP, Heng L, de Groot A, Gruppen H., 2007. Saponins, classification and occurrence in the plant kingdom. *Phytochemistry*. 68:275–297. https://doi.org/10.1016/j.phytochem.2006.10.008

Volkering, F., Breure, A. M., & Johan, G., 1995. Influence of Nonionic Surfactants on Bioavailability and Biodegradation of Polycyclic Aromatic Hydrocarbons. *Appl. Environ. Microbiol.*, 61(5), 1699–1705.

Von Lau E., Gan S., Ng H. & Poh P., 2014. Extraction agents for the removal of polycyclic aromatic hydrocarbons (PAH) from soil in soil washing technologies. *Environ. Pollut.*, 184, 640-649. https://doi.org/10.1016/j.envpol.2013.09.010

Part 1

Could saponins be used to enhance bioremediation of polycyclic aromatic hydrocarbons in aged-contaminated soils?

1. Foreword

The data presented in this section were acquired during the testing of a few experimental setups.

As explained previously, commercial saponin from *Quillaja saponaria* Molina bark, a natural surfactant, was purchased and used both in a soil extraction experiment and in a soil incubation experiment.

Several sets of data were collected through two experiments. The first experiment was the extraction (or washing) of an aged-contaminated soil with saponin solutions of increasing concentrations (0; 1; 2; 4 and 8 g L^{-1}). At the end of this extraction, the PAHs concentrations in the extracting solutions were measured based on an ISO norm (ISO 17993:2002) and constitute the first dataset. The second experiment was the incubation for 14 or 28 days of the same aged-contaminated soil, in microcosms (28°C), after the soil samples were amended with saponin (0; 2.5 and 5 mg g^{-1} DW). CO_2 emissions were monitored throughout the incubation (1, 3, 7, 14, 21, and 28 days) and soil dehydrogenase activities were measured after each incubation period, respectively constituting the second and third datasets. Finally, residual and bioaccessible PAHs contents were measured at the end of each incubation period. The PAHs residual (or total extractable) contents were also acquired using an ISO norm (ISO 13877:1998) and constitute the fourth dataset. In order to acquire the PAHs bioaccessible contents (the fifth dataset), a protocol had to be adapted to the specific experimental soil, since an ISO norm was not available at the time. It is important to mention that an ISO norm dedicated to the bioaccessibility measurement of hydrophobic compounds in soil was published in the course of the year 2018 (ISO/TS 16751:2018) and that the protocol that was applied in this thesis is very similar to the ISO norm, as will be discussed later.

The first four datasets (i.e. PAHs contents in aqueous saponin solutions, CO_2 emissions, dehydrogenase activities, and PAHs residual contents) were discussed in a publication, exposed hereafter (Davin *et al.*, 2018). The PAHs bioaccessible contents (the fifth dataset) will be discussed at the end of the chapter, along with a few complementary figures summarizing the published data (complementary data and figures section).

Finally, and to complete the publication, a characterization of the surface-active properties of the commercial saponin was performed as the product's Critical Micellar Concentration (CMC) was determined.

Reference

Davin, M., Starren, A., Deleu, M., Lognay, G., Colinet, G., Fauconnier, M.-L., 2018. Could saponins be used to enhance bioremediation of polycyclic aromatic hydrocarbons in aged-contaminated soils? *Chemosphere*, 194, 414-421. https://doi.org/10.1016/j.chemosphere.2017.11.174

2. Abstract

Polycyclic aromatic hydrocarbons (PAH) are persistent organic compounds of major concern that tend to accumulate in the environment, threatening ecosystems and health. Brownfields represent an important tank for PAHs and require remediation.

Researches to develop bioremediation and phytoremediation techniques are being conducted as alternatives to environmentally aggressive, expensive and often disruptive soil remediation strategies.

The objectives of the present study were to investigate the potential of saponins (natural surfactants) as extracting agents and as bioremediation enhancers on an aged-contaminated soil. Two experiments were conducted on a brownfield soil containing 15 PAHs. In a first experiment, soil samples were extracted with saponins solutions (0; 1; 2; 4 and 8 g L^{-1}). In a second experiment conducted in microcosms (28°C), soil samples were incubated for 14 or 28 days in presence of saponins (0; 2.5 and 5 mg g^{-1}). CO_2 emissions were monitored throughout the experiment. After the incubation, dehydrogenase activity was measured as an indicator of microbiological activity and residual PAHs were determined. In both experiments PAHs were determined using High-Performance Liquid Chromatography and Fluorimetric Detection.

The 4 g L^{-1} saponins solution extracted significantly more acenaphtene, fluorene, phenanthrene, anthracene, and pyrene than water. PAHs remediation was not enhanced in presence of saponins compared to control samples after 28 days. However CO_2 emissions and dehydrogenase activities were significantly more important in presence of saponins, suggesting no toxic effect of these surfactants towards soil microbiota.

3. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous organic compounds that are brought in the environment through natural and anthropogenic incomplete combustions that occur during forest fires, industrial manufacturing, fossil fuel use, or waste incineration (Johnsen *et al.*, 2005). PAHs are composed of two or more condensed aromatic rings, and are characterized by high hydrophobicity and low aqueous solubility (Lakra *et al.*, 2013). Once emitted in the air or in water, those compounds can accumulate on solid phases, making soil and sediments the main receptor for hydrophobic contaminants in general. Furthermore, PAHs present multiple health-concerning properties such as mutagenicity, carcinogenicity or teratogenicity, explaining why they have been of major concern (Zhang *et al.*, 2006). They are classified in two main categories: the low molecular weight PAHs, including molecules bearing three rings or less (naphthalene, acenaphtene, fluorene, phenanthrene, and anthracene) and the high molecular weight PAHs, including molecules of four rings or more (fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoran-thene, benzo[a]pyrene, dibenzo[ah]anthracene, benzo[ghi]perylene, and indeno[123-c,d]pyrene) (Megharaj *et al.*, 2001; Von Lau *et al.*, 2014).

Many remediation strategies have been applied to contaminated soils but often they turn out to be environmentally aggressive, expensive and disruptive towards soil. Some techniques even tend to postpone the treatment of the pollutants by either confining or translocating them to another environmental compartment (air or water). Bioremediation is a process relying on microorganisms, plants or their respective enzymes to degrade pollutants (Megharaj *et al.*, 2001).

The bioremediation mechanisms are influenced by pollutants availability to soil microorganisms (and their degrading enzymes) and the microbiota global fit. The pollutants availability greatly depends upon their physico-chemical properties (e.g. aqueous solubility, hydrophobicity, and molecular structure). Environmental factors (like organic matter and clay minerals can chemically or physically segregate the compounds) influence this availability by decreasing the accessibility to degrading agents. Furthermore, interacting factors such as pH, salinity, water content, temperature, redox potential, and water-dissolved oxygen and mineral nutrients will provide conditions more or less favourable to the activity of the degrading agents (Masciandaro *et al.*, 2013).

The bioavailability number has been defined as “the rate of mass transfer of a compound to a microbial cell to the rate of uptake and metabolism i.e. the intrinsic activity of the cell” (Bosma *et al.*, 1997; Johnsen *et al.* 2005). Therefore, the biodegradation rate is mainly controlled by the mass transfer to the cell or by the cell activity when the ratio is respectively >1 or <1 (Johnsen *et al.*, 2005).

Surfactants are surface-active molecules of amphiphilic nature. When present in an aqueous solution, these compounds can associate into different structures, depending on their nature, their concentration, and abiotic conditions (pH, ionic force, occurrence of solid phases). When present in low concentrations, surfactants remain as monomers and place themselves at the interface between a hydrophobic and a hydrophilic phase (e.g. air and water). Surfactants form micelles (aggregates of monomers) above a defined concentration called critical micellar concentration (CMC) (Lakra *et al.,* 2013). This surfactant property has been widely investigated over the last decades in order to use surfactants in soil “washing technologies” (Von Lau *et al.,* 2014) or to increase mass transfer of contaminants towards degrading cells (Kobayashi *et al.,* 2012) by increasing the apparent solubility of PAHs in water. Finally it is noteworthy that when solid phases such as soil are present; surfactants can also aggregate into structures that adsorb onto particles. Two well-known structures are the hemimicelle (a single layer of monomers adsorbed on a solid phase) and the admicelle (similar to the hemimicelle but with a second layer of monomers bond to the first one) (Makkar and Rockne, 2003).

Saponins are a class of natural non-ionic surfactants that are largely distributed in higher plants. They are composed of a sapogenin (hydrophobic) skeleton of either steroidal or triterpenoidal nature coupled to a glycose (hydrophilic) moiety (Oleszek & Bialy, 2006). Even though saponins are nowadays frequently used in pharmaceutical and cosmetic industries, they originally were employed for their foaming property as natural detergents (Sparg *et al.,* 2004). Therefore, the potential of saponins to enhance PAHs solubilisation has been investigated in recent studies. Zhou *et al.* (2011) have shown that saponins derived from *Quillaja saponaria* Molina bark are more effective at enhancing apparent solubility of phenanthrene in water than synthetic non-ionic surfactants (Tween 80, Triton X-100 and Brij58) whereas Kobayashi *et al.* (2012) have demonstrated an increase of the apparent hydrosolubility of phenanthrene, pyrene, and benzo[a]pyrene. They also showed that both biodegradation of pyrene and growth of *Sphingomonas* sp were related to the occurrence of saponins. They concluded that saponins had no antimicrobial activity, in spite of some previous experiments reporting that some saponins were capable of inhibiting microbial growth of low-density populations (Killeen *et al.,* 1998). Finally the same authors reported a removal of freshly-spiked pyrene from soil samples presenting a low organic carbon content (<0.1 %) using aqueous solutions of saponins.

The objective of the study presented herein was to investigate the possibility of using saponins as extracting agent and as bioremediation enhancer on an aged-contaminated soil containing several PAHs. Therefore, two experiments were conducted on a brownfield soil presenting 15 PAHs of interest. The first experiment was conducted to determine whether saponins solutions could extract more PAHs compounds than distilled water. Several concentrations of saponins were tested and extracted concentrations of the 15 PAHs were determined and compared. In the second experiment, contaminated soil was treated with saponins and incubated. Two concentrations of saponins and two incubation periods were tested. Several parameters were examined: (i) the carbon dioxide emission was monitored during the incubation process; (ii) the soil dehydrogenase activity was determined at the end of the incubation period as an indicator of saponins' toxicity towards the microbiota; and (iii) the residual PAHs contents were determined on soil samples after each incubation period.

4. Materials and methods

4.1. Soil material

The aged-contaminated soil used for this study was sampled on a brownfield in Saint-Ghislain, Belgium in a former coking plant which has been exposed for 70 years to petroleum hydrocarbons, PAHs, cyanides and trace elements. The particle size distribution (81.1 % sand, 10.7 % silt, 8.2 % clay) identified the soil as loamy sand. Other characteristics were pH_{H_2O} = 6.7 (according to ISO 10390:2005), total organic carbon (according to Springer and Klee, 1954), was 9.44 ± 0.22 % (W/W), and total nitrogen content (according to Bremner, 1982), was 0.16 ± 0.02 % (W/W). Soil was sampled, allowed to dry at ambient air, sieved through a 2-mm sieve and stored in sealed boxes until further use. Before the experiments, the contents of 15 PAHs were determined to range from 2.9 ± 0.1 mg kg^{-1}DW to 65.9 ± 7.1 mg kg^{-1}DW (Table 4). The compounds were naphthalene (N), acenaphtene (Ace), fluorene (Fle), phenanthrene (Phen), anthracene (Anthr), fluoranthene (F), pyrene (Pyr), benzo[a]anthracene (BaA), chrysene (Chrys), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP), dibenzo[ah]anthracene (DBahA), benzo[ghi]perylene (BghiP), and indeno[123-c,d]pyrene (IcdP). The Belgian Walloon legislation sets the reference value (i.e. the natural background) for each PAH in soils regardless of their occupation, at 0.01 mg kg^{-1}DW except N and Phen for which reference values are set at 0.1 mg kg^{-1}DW. This reference value (VR) is the ideal value to reach when there is a soil remediation. Depending on the soil's occupation (industrial, commercial, residential, agricultural or natural), different intervention values (VI: over which brownfield soils are to be systematically cleaned-up) and threshold values (VS: over which at least a risk assessment and a monitoring must be implemented) have been defined and are available in Supplementary table 1. The experimental soil shows PAHs contents higher than the threshold values for a commercial occupation for the 15 PAHs. All but F are also above the threshold values for an industrial occupation and N, Anthr, BaA, BbF, and BaP are above the intervention values for the industrial occupation (Décret relatif à la gestion des sols, 2009).

4.2. Saponins material and surface-active properties characterization

Crude extracts of saponins (batch number 14L190008) derived from *Quillaja saponaria* bark were purchased from VWR International (Leuven, Belgium) and used without further purification. The total organic carbon and the total nitrogen contents were 42.57 ± 0.22 % and 0.13 ± 0.02 % (W/W) respectively.

The CMC was determined using a Langmuir Kibron film balance composed with a 20 mL teflon tank and a rod used to measure surface pressures. Increasing solutions of raw saponins were prepared in dimethylsulfoxide (DMSO) by dilution of a 100 g L^{-1} stock solution. 15 µL of solution were injected in ultrapure water (15 mL) in order to reach concentrations from 1 mg L^{-1} to 100 mg L^{-1} in the subphase. Changes in surface pressure were recorded until they reached a plateau. The same volume of pure DMSO was injected in the subphase and no change of surface pressure was observed. The measures were taken at a temperature of 25°C. When plotting the evolution of the maximal surface pressure as a function of the saponins concentration, the CMC is the point at which the surface pressure no longer increases with the concentration. This point was determined as the intersection of two linear regression lines: one fitting the ascending part and one fitting the plateau, as described by Gatard *et al.,* 2013.

4.3. Experimental devices

Extraction experiments

Extraction experiments were conducted in glass flasks. Saponins solutions were prepared in water above the CMC, at respectively 1, 2, 4 and 8 g L^{-1} and tested as extracting solutions. Distilled water was used as a control. Each extraction was repeated five times. Briefly, 5 g of dry experimental soil were placed at 80 % of water holding capacity and extracted using magnetic stirring with 10 mL of aqueous solution for 24 h, in the dark. The aqueous phase was recovered by filtration. Results related to soil samples extracted by 1, 2, 4 and 8 g L^{-1} of saponins solutions have been named Sap1, Sap2, Sap4 and Sap8, respectively.

Incubation experiments

Incubation experiments were conducted in microcosms according to the norm AFNOR XP U44-163. Soil humidity conditions were chosen according to Barnier (2009) and Louvel (2010). Briefly, 15 g of dry experimental soil were placed at 80 % of water holding capacity and allowed to pre-incubate for 3 days. Once saponins were added to samples, two vessels were placed next to each sample in a sealed jar. One vessel was filled with distilled water to prevent soil desiccation and one was filled with NaOH solution to control carbon dioxide emission. Jars were incubated in the dark, at 28°C. At the end of the incubation period, soils were sacrificed for dry weight, dehydrogenase activity and PAHs measurements. Saponins were added to the soil samples in order to reach concentrations of 2.5 mg g^{-1}DW or 5 mg g^{-1}DW respectively. Those amendments are a compromise both to the norm AFNOR XP U44-163, limiting the organic carbon amended to a soil to 2 ‰ of the soil dry weight, and to soil composting recommendations to observe a C/N ratio between 100 : 5 and 300 : 5 (Colombano *et al.,* 2010). Untreated soils served as controls and two incubation periods (14 and 28 days) were investigated. All modalities were repeated four times for a total of 24 samples. Results related to soil samples with 2.5 and 5 mg saponins.g^{-1}DW have been named Sap2.5 and Sap5, respectively.

4.4. *Chemical analyses*

Dry weight determination

Soil samples dry weight determination was based on ISO 11465:1993 cor 1994.

Carbon dioxide emission

Carbon dioxide emission was monitored for each soil sample throughout the whole incubation following a method described in AFNOR XP U44-163. A vessel containing 15 mL of 0.5 M NaOH was placed in each jar as a carbon dioxide trap. Remaining NaOH was measured using automated pH-metric back-titration by acid (1 M). Before titration, barium chloride was added to precipitate carbonates. The equivalence point was set at pH 8.6. CO_2 emissions were measured after 1, 3, 7, 14, 21 and 28 days of incubation. Each time, fresh NaOH solution was replaced in the vessel and a blank was analysed to subtract ambient CO_2 from the measures. CO_2 emissions have been expressed in mg CO_2 g^{-1}DW.

Dehydrogenase activity

Dehydrogenase activity was measured for each soil sample after the incubation following a method described by Shaw and Burns (2005). Each sample was split in two sub-samples. Both were analysed the same way but one was previously sterilised by 3 cycles of 20 min at 121°C. One gram of fresh soil sample (sterilised or not) was added with 4 mL of iodonitrotetrazolium chloride 0.2 % (W/V) and incubated 48 h at 25°C in a sealed container. Samples were extracted with 10 mL of a 50:50 (V/V) N,N-dimethylformamide: ethanol mixture, centrifuged and the iodonitrotetrazolium formazan (INTF) produced by the enzymatic reduction was detected spectrophotometrically at 464 nm. INTF quantification was realised using external standard calibration. The signals measured for the sterilised samples served as blanks and were substracted from the regular sample signals. Dehydrogenase activity is expressed in µg INTF g^{-1}DW $48h^{-1}$.

PAHs determination in aqueous samples

PAHs determination in the aqueous samples was based on ISO 17993:2002. The aqueous phase was extracted twice with n-hexane during 1 h, and separated in a funnel. The organic phase was dried on anhydrous Na_2SO_4, eliminated with a rotative evaporation device, and replaced with acetonitrile. The final extract was weighed for volume determination and analysed for PAHs.

PAHs determination in soil samples

PAHs determination in soil samples was based on ISO 13877:1998. Briefly, soils were dried with an equivalent amount of anhydrous Na_2SO_4 and homogenised. The mixture was extracted with dichloromethane on a Soxhlet device for 16 h. The resulting organic phase was filtered on anhydrous Na_2SO_4, eliminated with a rotative evaporation device and replaced with n-hexane. Then the extract was purified on basic aluminium oxide before n-hexane was eliminated with a rotative evaporation device, and replaced by acetonitrile. The final extract was weighed for volume determination and analysed for PAHs.

PAHs analysis

PAHs (20 µL of acetonitrile extract) were injected on an Agilent reverse-phase C18 column (Eclipse PAH 4.6 X 250 mm, 5 µm) with external guard column (Eclipse PAH 4.6 X 12.5 mm, 5 µm) using a mixture of acetonitrile and water as eluents. Both mobile phases were acidified with formic acid (0.1% V/V). The separation was performed at a constant 1.5 mL min^{-1} flow rate using the following optimized gradient with the acetonitrile/water ratios: 0-15 min, linear increase from 50:50 to 75:25; 15-20 min, linear increase from 75:25 to 100:0; 20-40 min, 100:0. Finally: 40-40.1 min, linear decrease from 100:0 to 50:50 with a final isocratic hold of 5 min. PAHs were detected fluorimetrically according to ISO 13877:1998 and their quantification has been achieved using external standard calibration.

Statistics

All statistical analysis was carried out using Minitab 17.0. Data were analysed by general linear model or one-way analysis of variance and mean values were compared by Tukey’s test at the 5 % confidence level.

5. Results and discussion

5.1. Saponins CMC

Figure 2 shows the measured surface pressures for raw commercial saponins solutions. The first part of the graph shows a sharp increase of the surface pressure with the saponins concentration before reaching a plateau (second part). The intersection of the two parts is calculated to be 30.2 mg L^{-1} for a 26.9 mN m^{-1} surface pressure. As a comparison, Tween 80 (a synthetic nonionic surfactant) has a CMC of about 15 mg L^{-1} (Tween®80 product information) and the CMC of rhamnolipids (a type of biosurfactant produced by *Pseudomonas aeruginosa*) was reported at 150 mg L^{-1} (Gabet, 2009). The saponins solutions, prepared at 1, 2, 4 and 8 g L^{-1} and used in the extraction experiments thus ranged from 30 to 260 fold the CMC, meaning there were enough molecules to form micelles.

5.2. PAHs extractions by saponins

The extractions of soil samples by different saponins solutions (water, Sap1, Sap2, Sap4 and Sap8) allowed extracting PAHs contents ranging from 3 to 864 ng g^{-1}DW (Table 3). Statistical analyses show significant differences between the different extraction solutions for a few compounds.

When comparing each saponins solution to water, it appears that: (i) Sap2 extracted significantly more Ace, Fle, and Anthr than water; (ii) Sap4 extracted significantly more Ace, Fle, Phen, Anthr, and Pyr than water; and (iii) Sap8 extracted significantly more Fle, Phen, and Anthr than water.

When comparing, for one PAH, the saponins solutions that provided a significantly better extraction than water, it appears that: (i) Ace was significantly more extracted by Sap2 and Sap4 solutions, but there was no statistical difference between these two solutions; (ii) Fle and Anthr were significantly more extracted by Sap2, Sap4 and Sap8 but here again there was no statistical difference between the three solutions; (iii) Phen was significantly more extracted by Sap4 and Sap8, with no statistical difference between the two solutions; and (iv) Sap4 was the only solution that extracted significantly more Pyr than any other.

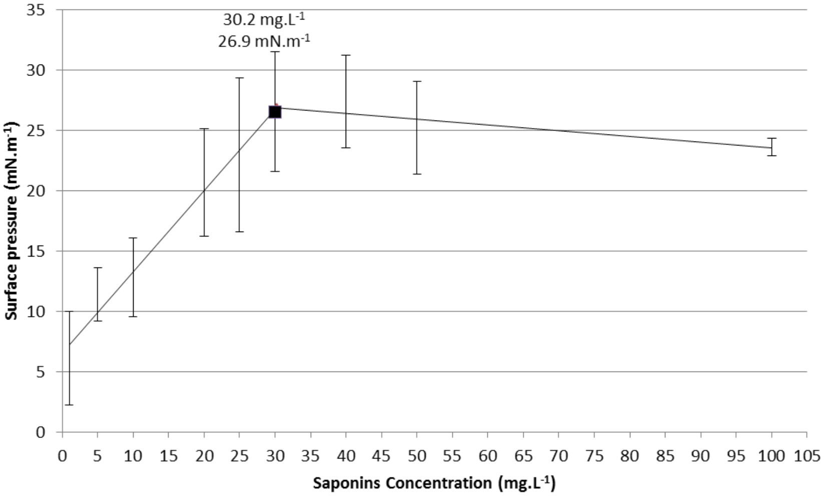


Figure 2. Determination of the critical micellar concentration of commercial *Quillaja saponaria* bark saponins saponins as the intersection of the two linear regression lines fitting the ascending part and the plateau. Values are means ± confidence interval.

Given the previous statements, it appears that the Sap4 solution is the best compromise among the different tested solutions as it allowed the extraction of the highest diversity of PAHs (Ace, Fle, Phen, Anthr, and Pyr).

It is interesting to examine the amounts extracted by the Sap8 solution. As it contained twice more surfactants than the Sap4 solution, Sap8 was expected to extract more PAHs than Sap4. However in some cases (Ace, Anthr, and Pyr) the statistical means structuration showed that not only were the extracted amounts not statistically different from Sap4 but also that they were not significantly different from water (Ace and Pyr) and from Sap1 (Anthr), meaning Sap8 provided a less efficient extraction than Sap4 for these compounds. Zhou *et al.* (2011) have determined that in aqueous conditions, the apparent solubilities of naphthalene, acenaphtylene (not detected in the present contamined soil), phenanthrene and pyrene increased linearly with the saponins concentration above the CMC. However, their tested saponins concentrations ranged from 1 to 25 fold the CMC (versus 30 to 260 fold the CMC in the present study) and their data does not show whether the PAHs solubilisation enhancements reach a maximum at higher saponins concentrations. Also, their experiments do not involve soil. Kobayashi *et al.* (2012) reported that an aqueous saponins solution with a concentration above the CMC significantly extracted pyrene from low organic carbon soil. However they used freshly pyrene-spiked soil. Haigh (1996) in her review on surfactants/soil/organic contaminants interactions mentions several factors that would prevent non-ionic surfactants to desorb hydrophobic compounds from soil particles. Hydrophobic interactions exist between soil particles and surfactants which could explain the lower extractions for Ace, Anthr, and Pyr by the Sap8 solution: the PAHs could be partitioned inside micelles, but the saponins constituting the micelles could bind to soil particles. Therefore, the benefit of the PAHs hydrosolubility being raised by the surfactants would be lost because the adsorption of the micelles to solids indirectly binds PAHs back to soil. This explanation could highlight a limitation to techniques that attempt to extract PAHs from soils by washing them with surfactants solutions: in some cases if the surfactant concentration is under or even close to the CMC, no desorption can be expected because monomers bond to soil particles are not capable of forming micelles, but if the surfactant concentration is too high, then micelles could raise the apparent sorption of the organic pollutants onto soil particles.

Table 3. PAHs extractions by different solutions (ng g^{-1} DW).

PAH	**Solution**					**p-value (α=0.05)**
	Water	**Sap 1g L^{-1}**	**Sap 2g L^{-1}**	**Sap 4g L^{-1}**	**Sap 8g L^{-1}**	
Naphthalene	$132^{a} \pm 31$	$203^{a} \pm 50$	$305^{a} \pm 85$	$294^{a} \pm 124$	$270^{a} \pm 128$	NS
Acenaphtene	$320^{b} \pm 85$	$539^{ab} \pm 173$	$818^{a} \pm 303$	$864^{a} \pm 121$	$706^{ab} \pm 254$	0.009
Fluorene	$106^{b} \pm 35$	$184^{ab} \pm 66$	$338^{a} \pm 136$	$354^{a} \pm 78$	$344^{a} \pm 118$	0.004
Phenanthrene	$129^{c} \pm 47$	$209^{bc} \pm 72$	$385^{abc} \pm 160$	$459^{ab} \pm 152$	$471^{a} \pm 151$	0.003
Anthracene	$41^{c} \pm 14$	$65^{bc} \pm 21$	$113^{ab} \pm 35$	$124^{a} \pm 22$	$119^{ab} \pm 33$	0.001
Fluoranthene	$101^{a} \pm 33$	$141^{a} \pm 41$	$202^{a} \pm 79$	$227^{a} \pm 41$	$225^{a} \pm 82$	0.027
Pyrene	$68^{b} \pm 17$	$103^{ab} \pm 43$	$135^{ab} \pm 49$	$167^{a} \pm 29$	$144^{ab} \pm 51$	0.024
Benz[a]anthracene	$26^{a} \pm 12$	$37^{a} \pm 9$	$44^{a} \pm 17$	$58^{a} \pm 19$	$55^{a} \pm 36$	NS
Chrysene	$30^{a} \pm 14$	$46^{a} \pm 12$	$51^{a} \pm 20$	$64^{a} \pm 19$	$63^{a} \pm 39$	NS
Benzo[b]fluoranthene	$37^{a} \pm 12$	$48^{a} \pm 23$	$55^{a} \pm 26$	$63^{a} \pm 26$	$47^{a} \pm 17$	NS
Benzo[k]fluoranthene	$12^{a} \pm 4$	$19^{a} \pm 8$	$17^{a} \pm 8$	$23^{a} \pm 8$	$20^{a} \pm 10$	NS
Benzo[a]pyrene	$20^{a} \pm 7$	$29^{a} \pm 14$	$27^{a} \pm 13$	$36^{a} \pm 11$	$28^{a} \pm 14$	NS
Dibenzo[ah]anthracene	$10^{a} \pm 5$	$9^{a} \pm 7$	$9^{a} \pm 8$	$15^{a} \pm 12$	$3^{a} \pm 3$	NS
Benzo[ghi]perylene	$14^{a} \pm 7$	$26^{a} \pm 14$	$17^{a} \pm 6$	$40^{a} \pm 48$	$19^{a} \pm 10$	NS
Indeno[1,2,3-cd]pyrene	$10^{a} \pm 5$	$19^{a} \pm 12$	$17^{a} \pm 6$	$15^{a} \pm 4$	$18^{a} \pm 8$	NS

Values are means ± confidence interval (n=5).

p-values (5% confidence level) indicate whether amounts of a PAH extracted by different solutions are significantly different (NS means differences are not significant).

Letters accolated to the values show Tukey’s means structuration groups.

5.3. PAHs bioremediation in the presence of saponins

Respiration curves and dehydrogenase activities

Figure 3 presents the CO_2 emissions of (un)treated soil samples during incubation. All samples show a rapid emission during the first two weeks of incubation then slow down towards a plateau. Cumulated emissions at days 14 and 28 are statistically different for the three incubation modalities and increase with the saponins content. One could hypothesize that the increase of the CO_2 emission is simply linked to the degradation of saponins. Nevertheless, assuming that all the saponins added to Sap2.5 and Sap5 samples had been completely degraded during the incubation, the maximal increase of CO_2 emission (calculated according to saponins carbon content) would be of respectively 0.26 and 0.52 mg CO_2 g^{-1}DW. However, the differences of CO_2 emitted after only 14 days of incubation between Sap2.5 or Sap5 samples and the control are respectively of 0.80 and 2.92 mg CO_2 g^{-1}DW which is about three to five times more. So the presence of saponins increases the global CO_2 emission to a greater extent than their degradation.

Figure 4 shows the dehydrogenase activity in the different (un)treated soil samples after 14 and 28 days of incubation. The activities of the control samples slowly decrease with time. On the other hand, soil samples treated with saponins show a sharp increase of their enzymatic activities during the first two weeks then a diminution during the next two weeks of incubation, regardless of the amended concentration. Besides, the dehydrogenase activity of Sap5 samples is about twice the activity of Sap2.5 samples and is statistically different at day 14. Dehydrogenase activity is a common indicator for soil biological activity (Das and Varma, 2011). Therefore it is reasonable to assume that the diminution of this activity, consistent with the slowing of CO_2 emission (Figure 3), represents the slowing of the global microbial activity in soil samples. Given the higher amounts of CO_2 emitted when saponins are supplied, an explanation is that this carbon source, being rapidly available for microorganisms, is rapidly metabolized and boosts the soil global activity until it starts to lack. At this moment (14 days) the enzymatic activity slows down along with the CO_2 emission. Therefore both CO_2 emission and dehydrogenase activity sets of data suggest that there is no toxic effect of the added saponins towards the soil microbiota.

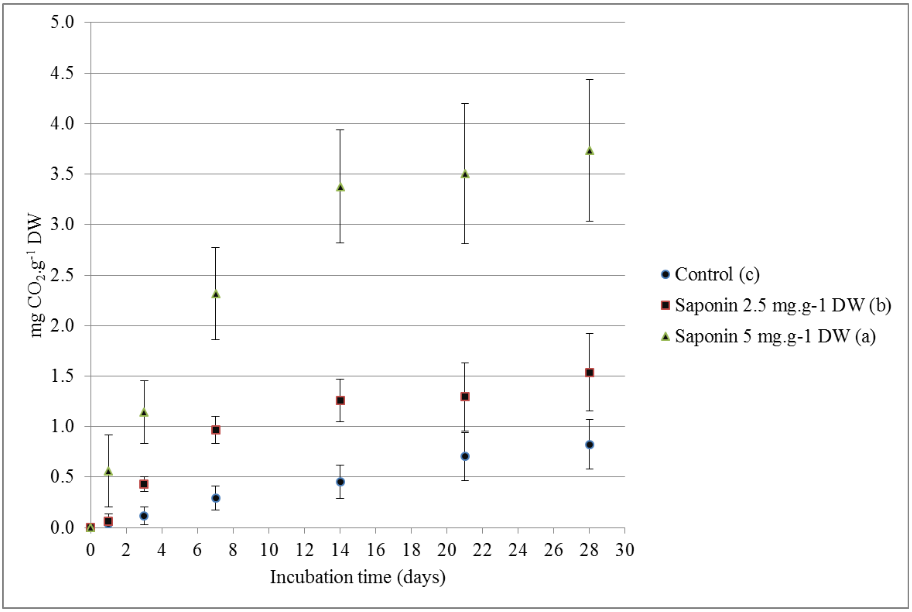


Figure 3. CO_2 emissions during the incubation of soils treated with saponins. Values are means ± confidence interval. Treatments followed by the same letter are not significantly different ($p > 0.05$).

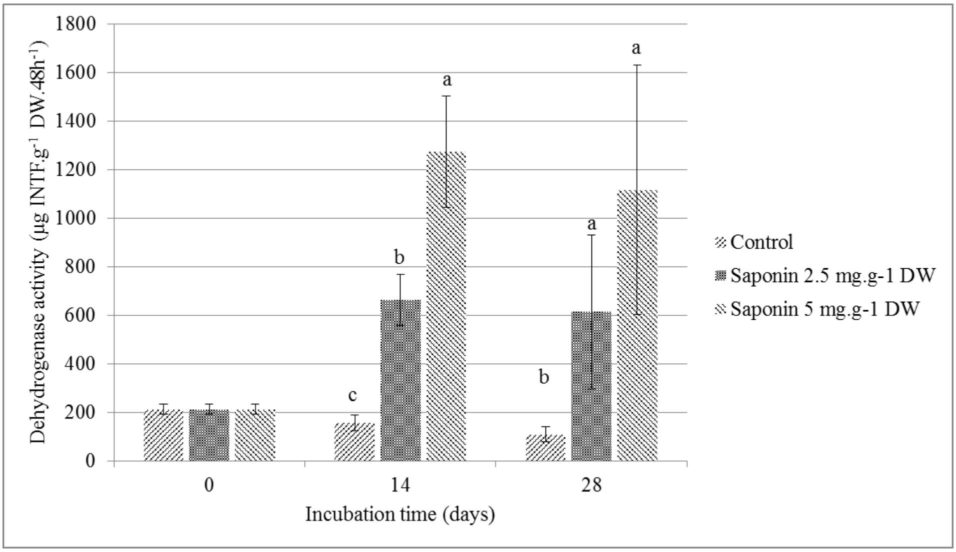


Figure 4. Dehydrogenase activity of the soils treated with saponins after different incubation periods. Values are means ± confidence interval. Within each time group, sticks with the same letter are not significantly different ($p > 0.05$).

PAHs residual contents

Residual PAHs contents of (un)treated soils after 14 and 28 days of incubation are presented in Table 4. Residual mean values, when compared to the values of the Belgian Walloon legislation norms available in Supplementary table 1, show that even though none of the incubation modalities were able to lower the PAHs down to their respective reference value (0.01 or 0.1 mg kg^{-1}DW), some compounds have been lowered enough to change soil occupation criteria.

A few observations can be made from examining each PAH residual mean after each incubation scenario: (i) in all incubation modalities N lowered under 25 µg.g^{-1}DW (industrial VI) and Ace under 19 µg.g^{-1}DW (residential VI) as soon as after 14 days of incubation; (ii) in control samples and after 14 days, Anthr reached 13.3 µg.g^{-1}DW (industrial VI); (iii) in control samples and after 28 days, Anthr passed under the industrial VI, Fle passed under 9 µg.g^{-1}DW (both residential and commercial VS) which is also under 26 and 16 µg.g^{-1}DW (natural and agricultural VIs, respectively), F passed under 47 µg.g^{-1}DW (industrial VS) and thus under 48 µg.g^{-1}DW (agricultural VI), and Chrys passed under 25 µg.g^{-1}DW (both residential and commercial VIs); and (iv) in Sap5 samples and after 28 days, Anthr passed under the industrial VI, and Fle under both the natural and agricultural VI.

Statistical analyses give complementary information: (i) when comparing the residual PAHs contents after either 14 or 28 days, it appears that the values in samples treated with saponins (both Sap2.5 and Sap5) are not statistically different from the control samples at any incubation time; and (ii) there is a significant effect of the time: N and Ace, on one hand, and Phen, F, and Pyr, on the other hand, are statistically different from the initial content after 14 and 28 days respectively. However in Sap2.5 and Sap5 samples this time-effect on the residual PAHs content is only observed for N and Ace whereas the controls also show such diminution for Phen, F, and Pyr. These observations point towards an inhibition of the PAHs disappearance in the presence of saponins rather than an enhancement.

When the experiment was imagined, it was based on the hypothesis that the addition of surfactants to an aged-contaminated soil would enhance PAHs remediation. Bouchez *et al.* (1995) demonstrated the capacity of PAHs-degrading bacterial strains to degrade some normally recalcitrant PAHs through co-metabolism pathways; Rentz *et al.* (2005) showed that the degradation of BaP by *Sphingomonas yanoikuyae* was enhanced in the presence of a primary, more available source of carbon such as salicylate or plant roots extracts; and finally Kobayashi *et al.* (2012) reported that the biodegration of pyrene by *Sphingomonas* sp. was enhanced in the presence of saponins. Similar events were expected in the present study but the results do not suggest likewise.

Zhu & Aithken (2010) conducted degradation experiments on aged-contaminated soil in the presence of two non-ionic synthetic surfactants: Brij® 30 (polyoxyethylene (4) lauryl ether: a hydrophobic surfactant) and $C_{12}E_8$ (octaethylene glycol mono *n*-dodecyl: a hydrophilic surfactant) and suggested the following conclusions: (i) the hydrophilic surfactant did not enhance PAHs degradation, at any concentration; and

(ii) in the presence of the hydrophobic surfactant, the degradation of 3-rings PAHs (such as Phen) rose with the surfactant concentration but the degradation of 4-rings PAHs (F and Pyr) was less enhanced at a surfactant concentration above the CMC. However no inhibition of the degradation process was mentioned. Also Tiehm (1994), in an attempt to enhance phenanthrene availability to *Mycobacterium* sp., in the presence of Phen and SDS (sodium dodecyl sulfate: a hydrophilic non-ionic synthetic surfactant) observed that the microorganisms metabolized SDS as a primary nutrient source instead of Phen. These observations are in line with the results of the present study which has given strong evidence that saponins are used as a carbon source instead of PAHs and that co-metabolism did not take place during the incubations. Indeed, even though the total organic carbon is increased by less than 2 %, the added carbon source (saponins) is much more available for biotransformation than PAHs.

The lower diminution of PAHs contents in the presence of saponins could also be related to the extraction results mentioned previously: if PAHs were secluded by saponins micelles or hemimicelles, either in the soil solution or adsorbed on soil particles, the pollutants would be less available for biodegradation.

Finally, it is important to bear in mind that given the higher surface tensions of N and Ace compared to the other compounds (10.5 Pa and 0.356 Pa at 25°C, respectively), their diminution with time in Sap2.5 and Sap5 samples might simply be a loss by volatilization. Such hypothesis would have to be verified by monitoring the gas emissions in the jar by solid phase micro-extraction sampling. Such case scenario would mean that only Phen, F, and Pyr are significantly degraded in the control samples and that the diminution of N and Ace in all samples (control, Sap2.5 and Sap5) is not significant.

Table 4. PAHs residual contents in soils treated with saponins and after different incubation times (mg kg^{-1} DW).

PAHs	**Initial**	**Control**		**Saponins 2.5mg g^{-1} DW**		**Saponins 5mg g^{-1} DW**	
		14 days	**28 days**	**14 days**	**28 days**	**14 days**	**28 days**
Naphthalene	28.9 ± 1.7	17.4 ± 1.0	18.1 ± 0.8	18.5 ± 4.9	21.4 ± 4.2	20.1 ± 0.8	16.7 ± 4.8
Acenaphtene	19.4 ± 1.2	12.2 ± 2.6	10.0 ± 1.4	14.4 ± 2.3	12.1 ± 2.7	13 ± 2.7	10.9 ± 2.8
Fluorene	12.5 ± 1.1	9.4 ± 1.4	8.4 ± 3.1	10.4 ± 0.8	10.6 ± 3.7	10.8 ± 3.0	8.7 ± 3.4
Phenanthrene	46.5 ± 5.5	37.2 ± 6.5	30.5 ± 2.9	38.1 ± 3.8	39.4 ± 11.9	40.6 ± 10.1	39.0 ± 9.4
Anthracene	16.0 ± 1.4	13.3 ± 2.4	11.7 ± 8.7	14.6 ± 1.0	16.1 ± 3.3	19.0 ± 5.9	12.4 ± 7.3
Fluoranthene	65.9 ± 7.1	55.1 ± 11.3	45.6 ± 5.9	53.4 ± 6.5	53.3 ± 8.2	53.7 ± 12.2	52 ± 10.3
Pyrene	45.6 ± 4.8	38.3 ± 1.3	34.4 ± 2.2	38.2 ± 1.0	38.0 ± 6.7	39.3 ± 5.0	38.0 ± 6.7
Benz[*a*]anthracene	28.3 ± 3.6	27.6 ± 3.4	22.8 ± 0.3	26.2 ± 0.4	27.4 ± 2.5	26.2 ± 2.4	27.6 ± 2.4
Chrysene	32.4 ± 4.0	32.9 ± 4.2	23.9 ± 13.9	31.1 ± 1.0	32.9 ± 3.1	31.6 ± 3.9	31.6 ± 6.4
Benzo[*b*]fluoranthene	23.1 ± 3.3	26.1 ± 5.8	19.6 ± 1.6	21 ± 0.8	22.0 ± 2.8	18.7 ± 2.2	22.1 ± 2.2
Benzo[*k*]fluoranthene	11.8 ± 1.6	10.7 ± 0.1	10.1 ± 0.5	10.8 ± 0.2	11.3 ± 1.0	10.7 ± 1.1	11.2 ± 1.0
Benzo[*a*]pyrene	18.3 ± 2.6	18.3 ± 2.4	17.3 ± 1.7	17.7 ± 0.2	19.4 ± 0.3	17.5 ± 1.5	19.2 ± 2.2
Dibenzo[*ah*]anthracene	2.9 ± 0.1	2.5 ± 0.8	2.3 ± 0.4	2.3 ± 0.5	2.4 ± 0.1	2.7 ± 0.3	2.7 ± 0.5
Benzo[*ghi*]perylene	14.1 ± 3.6	13.4 ± 1.6	11.2 ± 1.1	11.5 ± 0.9	12.6 ± 2.0	11.0 ± 1.0	11.4 ± 1.0
Indeno[1,2,3-*cd*]pyrene	15.0 ± 2.6	15.5 ± 2.6	14.4 ± 2.9	14.8 ± 1.0	16.2 ± 2.0	13.4 ± 2.1	13.8 ± 0.5

Values are means ± confidence interval (n=3 or 4).

6. Conclusions and perspectives

It is of major interest to extend the general research on PAHs bioremediation enhancement. One could imagine experiments similar to the ones previously describes (involving weathered soil and several PAHs) being carried out with other types of biosurfactants or plant-based amendments such as plant-root exudates, rhamnolipids, surfactin, humic and fulvic acids … However the purpose of the exposed extraction and incubation experiments was to evaluate the potential of saponins from *Quillaja saponaria* bark as a PAHs bioremediation enhancer by confronting this non-ionic surfactant to an aged-contaminated soil.

The extraction experiment has proven to be limited in efficiency as it has allowed the significant extraction of only a few compounds (Ace, Fle, Phen, Anthr, and Pyr). Besides, it seems that extraction decreases over a surfactant concentration threshold given the fact that a solution of 8g L^{-1} of saponins could statistically not extract higher amounts of PAHs than water (Ace and Pyr) or than a 1g L^{-1} solution of saponins (Anthr).

However this opens the debate towards the application of saponins in stabilization technologies. One could imagine that the present surfactant (saponins from *Quillaja saponaria* bark) could be used as a secluding agent that would help slowing down the migration of a fresh plume of pollution involving PAHs towards a sensitive compartment (such as groundwater) through the binding of PAHs to soil particles. Given the overall biodegradability of biosurfactants, such an application would be temporary and have to be associated to a more permanent treatment. Besides, complementary studies would have to be conducted because as reviewed by Haigh (1996), the interactions of surfactants strongly depend on the soil mineralogy and organic matter.

The incubation experiment results strongly suggest that the presence of saponins in the experimental soil has no enhancement effect on the PAHs bioremediation and even slows down this process. Therefore, there would be no advantage in treating a polluted soil with saponins from *Quillaja saponaria* bark during a bioremediation treatment.

On the other hand, the increase in the dehydrogenase activities and the higher emissions of carbon dioxide when soil was treated show that the saponins do not have a toxic effect on soil microbiota and even seem to increase its activity. Therefore it would be interesting to start over a similar experiment and conduct it for a longer time to assess whether the regular input of saponins could allow the soil microbial activity to last longer by regularly boosting the microbiota. Maybe such action would allow the PAHs remediation to be conducted on a longer period but in a more thorough way.

When crossing incubation and extraction results, two main hypotheses stand out that would explain the greater diminution of PAHs contents in the absence of saponins: (i) the surfactant is preferably degraded over the pollutants; and (ii) the surfactants partitioned the available PAHs into micelles, making them less bioavailable to biodegradation. The first hypothesis would have to be verified by implementing a cell culture similar to the one realised by Tiehm (1994) to assess whether PAHs-degraders could use saponins from *Quillaja saponaria* bark as primary nutrients over PAHs and the second by evaluating the bioavailability of PAHs in the presence of saponins through the use of Tenax® beads for example (Cornelissen *et al.*, 2001).

The conclusion that stands out from the results and interpretations exposed in the present article is that saponins from *Quillaja saponaria* bark, if they were added to an aged-contaminated soil in the tested concentrations, would not enhance PAHs bioremediation in the short run (28 days).

7. References

AFNOR XP U44-163 Amendements organiques et supports de culture - Caractérisation de la matière organique par la minéralisation potentielle du carbone et de l’azote.

Barnier, C., 2009. *Disponibilité des HAP dans les sols de friches industrielles et influence des conditions rhizosphériques.* PhD thesis : University of Nancy (France).

Bouchez, M., Blanchet, D., & Vandecasteele, J-P., 1995. Degradation of polycyclic aromatic hydrocarbons by pure strains and by defined strain associations: inhibition phenomena and cometabolism. *Appl. Microbiol. Biotechnol.*, 43, 156-164.

Bosma, T., Middeldorp, P., Schraa, G., Zehnder, A., 1997. Mass transfer limitation of biotransformation°: quantifying bioavailability. *Environ. Sci. Technol.*, 31, 248-252.

Bremner, J., & Mulvaney, C., 1982. Nitrogen-total, *In*: Page L., eds. *Methods of Soil Analysis, Part 2, Chemical and Microbiological Properties.*American Society of Agronomy, Inc., American Science Society of America, Inc., Madison, 595-624.

Colombano, S., Saada, A., Guerin, V., Bataillard, P, Bellenfant, G., Beranger, S., Hube, D., Zornig, C, Giradeau, I., 2010. Quelles techniques pour quels traitements ? Analyse coûts-bénéfices - Rapport final BRGM-RP-58609-FR

Cornelissen, G., Rigterink, H., ten Hulscher, D.E., Vrind, B.A., van Noort, P.C., 2001. A simple Tenax® extraction method to determine the availability of sediment-sorbed organic compounds. *Environ. Toxicol. Chem.*, 4, 706-711.

Das, S., & Varma, A., 2011. Roles of enzymes in maintaining soil health. *In*°: Shukla G. & Varma A., eds. *Soil enzymology*. Springer, 25-42.

Décret relatif à la gestion des sols (Moniteur Belge du 18/02/2009, p. 14852. Add.: Moniteur Belge du 06/03/2009, p. 19935)

Gabet, S., 2009. *Remobilisation d’hydrocarbures aromatiques polycycliques (HAP) présents dans les sols contaminés à l’aide d’un tensioactif d’origine biologique.* PhD thesis : University of Limoges (France).

Gatard, S., 2013. Bolaamphiphiles derived from alkenyl L-rhamnosides and alkenyl D-xylosides: importance of the hydrophilic head. *Molecules*, 18, 6101-6112.

Haigh, S., 1996. A review of the interaction of surfactants with organic contaminants in soil. *Sci. Total Environ.*, 185 (1-3), 161-170.

ISO 10390:2005 - Soil quality - pH determination.

ISO 11465:1993 cor 1994 - Soil quality - Determination of dry matter and water content on a mass basis - Gravimetric method.

ISO 13877:1998 Soil quality - Determination of polynuclear aromatic hydrocarbons - Method using high -performance liquid chromatography.

ISO 17993:2002 Water quality - Determination of 15 polycyclic aromatic hydrocarbons (PAH) in water by HPLC with fluorescence detection after liquid-liquid extraction.

Johnsen, A., Wick, L., & Harms, H., 2005. Principles of microbial PAH-degradation in soil. *Environ. Pollut.*, 133(1), 71-84.

Killeen, G., Madigan, C., Connolly, C., Walsh, G., Clark, C., Hynes, M., Timmins, B., James, P., Headon, D., Power, R., 1998. Antimicrobial saponins of Yucca schidigera and the implications of their in vitro properties for their in vivo impact. *J. Agr. Food Chem.,*46, 3178–3186.

Kobayashi, T., Kaminaga, H., Navarro, R., Iimura, Y., 2012. Application of aqueous saponin on the remediation of polycyclic aromatic hydrocarbons-contaminated soil. *J. Environ. Sci. Health. A.* 47, 1138-1145.

Lakra, J., Tikariha, D., Yadav, T., Satnami, M.L., Ghosh, K.K., 2013. Study of solubility efficiency of polycyclic aromatic hydrocarbons in single surfactant systems. *J. Surfact. Deterg.* 16, 957-966.

Louvel, B., 2010. *Étude en microcosmes de l'effet du ray-grass et de ses exsudats racinaires sur la dissipation des HAP et les communautés bactériennes dégradantes.* PhD thesis : University of Nancy (France).

Makkar, R., & Rockne, K., 2003. Comparison of synthetic surfactants and biosurfactants in enhancing biodegradation of polycyclic aromatic hydrocarbons. *Environ. Toxicol. Chem.,* 22(10) 2280-2292.

Masciandaro, G., Macci, C., Peruzzi, E., Ceccanti, B., Doni, S., 2013. Organic matter-microorganism-plant in soil bioremediation: A synergic approach. *Rev. Environ. Sci. Biotechnol*., 12, 399-419.

Megharaj, M., Ramakrishnan, B., Venkateswarlu, K., Sethunathan, N., Naidu, R., 2011. Bioremediation approaches for organic pollutants°: a critical perspective. *Environ. Int.,* 37, 1362-1375.

Oleszek, W., & Bialy, Z., 2006. Chromatographic determination of plant saponins—An update (2002–2005). *J. Chromatogr. A,* 1112, 78-91.

Rentz, J., Alvarez, P., & Schnoor, J., 2005. Benzo[a]pyrene co-metabolism in the presence ofplant root extracts and exudates: Implications for phytoremediation. *Environ. Pollut.,* 136, 477-484.

Shaw, L., & Burns, R., 2005. Soil microbial activity. *In*: Bloem J., Hopkins D.W. & Benedetti A., eds. *Microbiological methods for assessing soil quality*. Cambridge, MA, USA°: CABI, 114-182.

Tween®80 product information http://www.sigmaaldrich.com/catalog/product/sigma/p4780?lang=fr&region=BE (consulted 03/10/17)

Sparg, S., Light, M., & van Staden, J., 2004. Biological activities and distribution of plant saponins. *J. Ethnopharmacol.,* 94, 219-243.

Springer, U., & Klee, J, 1954. Prüfung der leistungsfähigkeit von einigen wichtigeren verfahren zur bestimmung des kohlenstoffs mittels chromschwefelsäure sowie vorschlag einer neuen schnell methode. *J. Plant Nutr. Soil Sc.,* 64, 1-26.

Tiehm, A., 1994. Degradation of polycyclic aromatic hydrocarbons in the presence of synthetic surfactants. *Appl. Environ. Microbiol.,* 258-263.

Von Lau, E., Gan, S., Ng, H., Poh, P., 2014. Extraction agents for the removal of polycyclic aromatic hydrocarbons (PAH) from soil in soil washing technologies. *Environ. Pollut.,* 184, 640-649.

Zhang, X. Cheng, S., Zhu, C., Sun, S-L., 2006. Microbial PAH-degradation in soil°: degradation pathways and contributing factors. *Pedosphere*, 16, 555-565.

Zhou, W., Yang, J., Lou, L., Zhu, L., 2011.Solubilization properties of polycyclic aromatic hydrocarbons by saponin, a plant-derived biosurfactant. *Environ. Pollut.,* 159, 1198-1204.

Zhu, H., & Aitken, M., 2010. Surfactant-enhanced desorption and biodegradation of polycyclic aromatic hydrocarbons in contaminated soil. *Environ. Sci. Technol.,* 44(19), 7260-7265.

8. Supplementary material

Supplementary table 1. PAHs norms in brownfield soils in the Wallon region (in the former Décret relatif à la gestion des sols, 2009).

Occupation		**Soil (mg/kg$_{DW}$)**				
		natural	**agricultural**	**residential**	**recreational or commercial**	**industrial**
Naphthalene (N)	VR	0.1	0.1	0.1	0.1	0.1
	VS	1.1	0.7	1.7	1.7	2.5
	VI	4	2.5	9	9	25
Acenaphtylene (A)	VR	0.01	0.01	0.01	0.01	0.01
	VS	0.3	0.3	0.8	8	43
	VI	3	3	8	78	410
Acenaphtene (Ace)	VR	0.01	0.01	0.01	0.01	0.01
	VS	2.6	1.6	3.9	3.9	6
	VI	9	6	19	19	56
Fluorene (Fle)	VR	0.01	0.01	0.01	0.01	0.01
	VS	4	2	9	9	16
	VI	26	16	46	46	163
Phenanthrene (Phen)	VR	0.1	0.1	0.1	0.1	0.1
	VS	9	6	12	12	16
	VI	27	16	60	60	164
Anthracene (Anthr)	VR	0.01	0.01	0.01	0.01	0.01
	VS	0.3	0.2	0.7	0.7	1.3
	VI	2.2	1.3	3.7	3.7	13.3
Fluoranthene (F)	VR	0.01	0.01	0.01	0.01	0.01
	VS	8	5	23	23	47
	VI	77	48	126	126	475
Pyrene (Pyr)	VR	0.01	0.01	0.01	0.01	0.01
	VS	1.4	0.9	3.6	3.6	6.4
	VI	10	6	18	18	64
Benzo[a]anthracene (BaA)	VR	0.01	0.01	0.01	0.01	0.01
	VS	0.8	0.5	1	1	1.5
	VI	2.5	1.5	5	5	15
Chrysene (Chrys)	VR	0.01	0.01	0.01	0.01	0.01
	VS	5	3	5	5	6
	VI	10	6	25	25	60

(continued)

Occupation		**Soil (mg/kg$_{DW}$)**				
		natural	**agricultural**	**residential**	**recreational or commercial**	**industrial**
Benzo[b]fluoranthene (BbF)	VR	0.01	0.01	0.01	0.01	0.01
	VS	0.7	0.4	0.3	0.9	1.3
	VI	2	1.5	4	4	13
Benzo[k]fluoranthene (BkF)	VR	0.01	0.01	0.01	0.01	0.01
	VS	2.5	1.6	1.3	3.1	4.7
	VI	7.6	4.7	12.8	15.5	47
Benzo[a]pyrene (BaP)	VR	0.01	0.01	0.01	0.01	0.01
	VS	0.2	0.2	0.5	0.9	1.3
	VI	2.2	1.3	4.5	4.5	13
Dibenzo[ah]anthracene (DBahA)	VR	0.01	0.01	0.01	0.01	0.01
	VS	0.8	0.1	0.6	1	1.4
	VI	2.3	0.7	5	5	14
Benzo[g,h,i]perylene (BghiP)	VR	0.01	0.01	0.01	0.01	0.01
	VS	2.5	1.5	3	3	5
	VI	7	5	15	15	46
Indeno[1,2,3-c,d]pyrene (IcdP)	VR	0.01	0.01	0.01	0.01	0.01
	VS	1	0.6	0.2	1.2	1.5
	VI	2.5	1.5	2.5	6	15

VR (Reference Value): ideal value to reach when there is a soil remediation

VS (Threshold value): over which at least a risk assessment and a monitoring must be implemented

VI (Intervention value): over which brownfield soils are to be systematically cleaned-up

9. Complementary data and figures

The data on the effect of commercial saponin from *Quillaja saponaria* Molina bark on PAHs bioaccessibility will be presented and examined, along with a few complementary figures summarizing previously exposed data.

9.1. Influence of commercial saponin on PAHs bioaccessibility

As a reminder, soil samples were incubated in microcosms (28°C) for 14 or 28 days in presence of commercial saponins (0, 2.5 and 5 mg g^{-1} DW). CO_2 emissions, soil dehydrogenase activities, and PAHs residual contents were discussed previously.

Figure 5 exposes the PAHs bioaccessible contents that were measured in soil samples, at the end of each incubation period. The protocol (further exposed in Part 2) was based on a 48 h Tenax® beads extraction of fresh soil samples. The presented data focusses on different groups of PAHs (∑2-3 rings, ∑4 rings, ∑4-6 rings, and ∑all), as they summarize and emphasize the observations made on individual PAHs. The PAHs group members are N, Ace, Fle, Phen, and Anthr for the ∑2-3 rings group; F and Pyr for the ∑4 rings group; BaA, Chrys, BbF, BkF, BaP, DBahA, BghiP, and IcdP for the ∑4-6 rings group; and N to IcdP for the ∑all group.

Also, please keep in mind that the soil samples were pre-incubated for 3 days before the amendments were added, thus there are two bioaccessible contents measured on the untreated soil before and after three days of pre-incubation, respectively named “-3 days” and “0 days” in Figure 5.

Statistical analyses on bioaccessible contents were performed after squareroot transformation. Analysis of variance showed significant interactions between time (-3, 0, 14, and 28 days) and treatment (C, Sap2.5, or Sap5) on ∑2-3 rings bioaccessible contents only. For the ∑4 rings, ∑4-6 rings, and ∑all bioaccessible contents, the analysis of variance showed a time effect.

Significant differences appear between the bioaccessible contents at “-3 days” and “0 days” for all groups of PAHs. Depending on the group and on the treatment, bioaccessible contents are 1.5 to 5-fold the ones measured initially (-3 days).

At the end of each incubation period (14 or 28 days), it is interesting to notice that similar groups of PAHs bioaccessible contents exhibit similar patterns, regardless of the type of treatment they received. After rising throughout the pre-incubation, all bioaccessible contents significantly lower back to pre-incubation levels or even under. However, in all cases, the bioaccessible contents after 28 days are not significantly different from the contents after 14 days. This clearly suggests that the amendments had no effect on the PAHs bioaccessible contents, compared to untreated samples.

9.2. Influence of commercial saponin on PAHs dissipation

Figure 6 exposes the PAHs residual contents that were measured in soil samples after 14 or 28 days of incubation in presence of commercial saponins. The data summarizes the results for each PAHs group (∑2-3 rings, ∑4 rings, ∑4-6 rings, and ∑all). As it emphasizes the observations made on individual PAHs, this figure is a complement to the PAHs residual data exposed in Table 4 that only presented individual PAHs contents. Analysis of variance showed a time effect on all PAHs residual groups, except the ∑4-6 rings group.

At the end of each incubation period (14 or 28 days), similar groups of PAHs residual contents display similar patterns, regardless of the type of treatment they received. Except for the ∑4-6 rings group, all PAHs residual contents are significantly lower than the initial content by the end of each incubation period. But in all cases, the residual contents after 28 days are not significantly different from the contents after 14 days. Such patterns show that the saponin amendments had no effect on the PAHs dissipation, compared to untreated samples.

9.3. Influence of commercial saponin on PAHs extraction

Figure 7 shows the PAHs contents that were extracted from aged-contaminated soil by several commercial saponin solutions (0, 1, 2, 4 or 8 g L^{-1}). The data emphasizes the observations made on individual PAHs in Table 3 as it summarizes the results for each PAHs group (∑2-3 rings, ∑4 rings, ∑4-6 rings, and ∑all). The graphs confirm the observations that were previously made on individual PAHs. Saponin solutions of 2, 4, and 8 g L^{-1} extracted more of the light (∑2-3 rings) and intermediate (∑4 rings) PAHs than water. This enhanced extraction appears also in the total extracted PAHs (∑all), but not in the heavy (∑4-6 rings) PAHs.

As was previously mentioned, the extracted PAHs contents in these three groups of PAHs (∑2-3 rings, ∑4 rings, and ∑all) tend to increase with the saponin's concentration until it reaches 4 g L^{-1}, then the extracted contents slightly decrease with the 8 g L^{-1} solution. Even though contents extracted by solutions of 4 and 8 g L^{-1} solutions are not statistically different, ∑2-3 rings, ∑4 rings, and ∑all extracted contents are systematically smaller in Sap8 samples than in Sap4 samples, as was noted for some individual PAHs (Table 3). This confirms the suggestion that when the surfactant's concentration is too high, saponins act as a secluding agent rather than a solubilizing agent and prevent PAHs from being extracted in aqueous solutions.

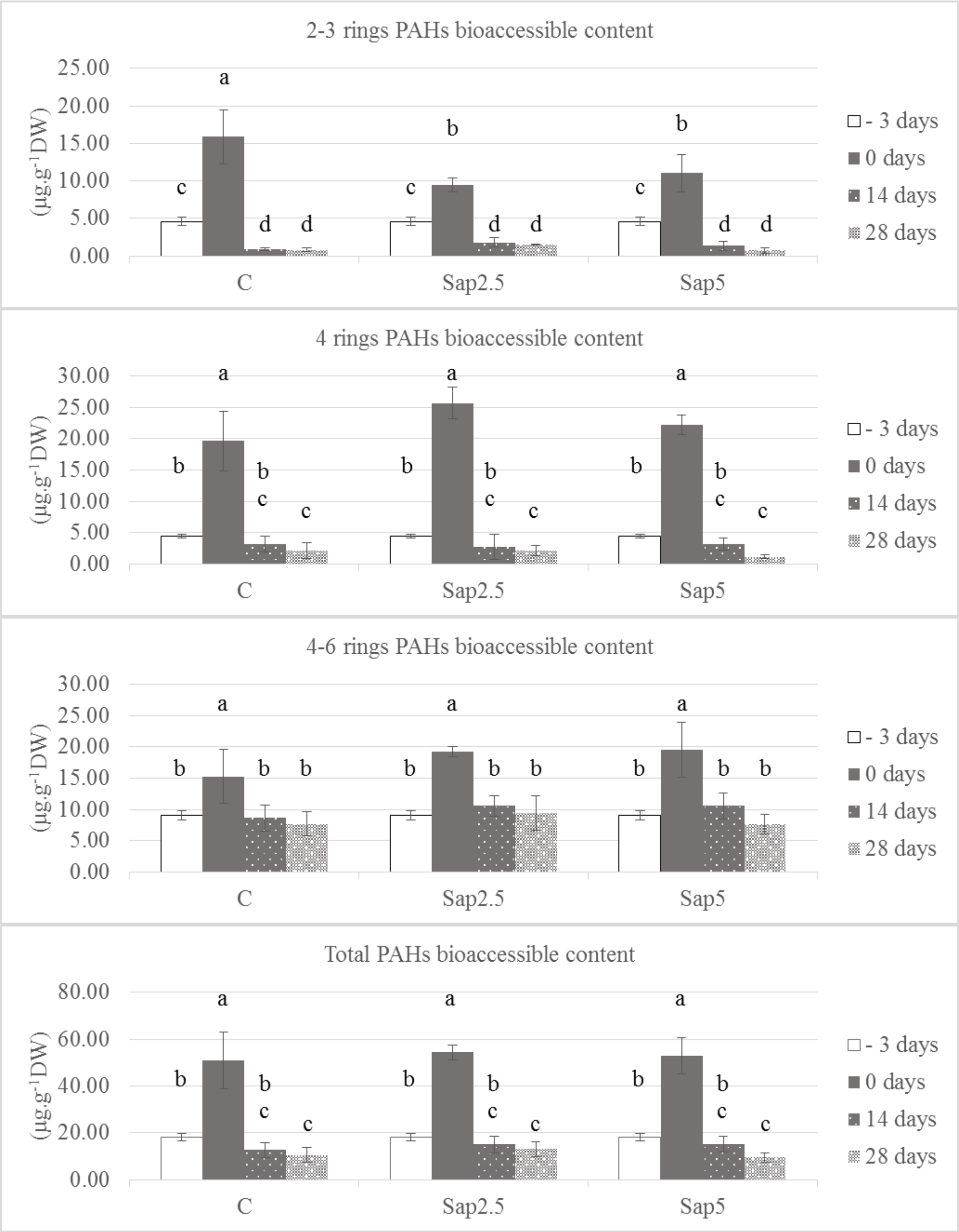


Figure 5. PAHs bioaccessible contents of soils treated with commercial saponin (0, 2.5 or 5 mg g^{-1} DW) and after different incubation times. Values are means ± confidence interval (α=5%). Sticks that share the same letter are not significantly different ($p > 0.05$).

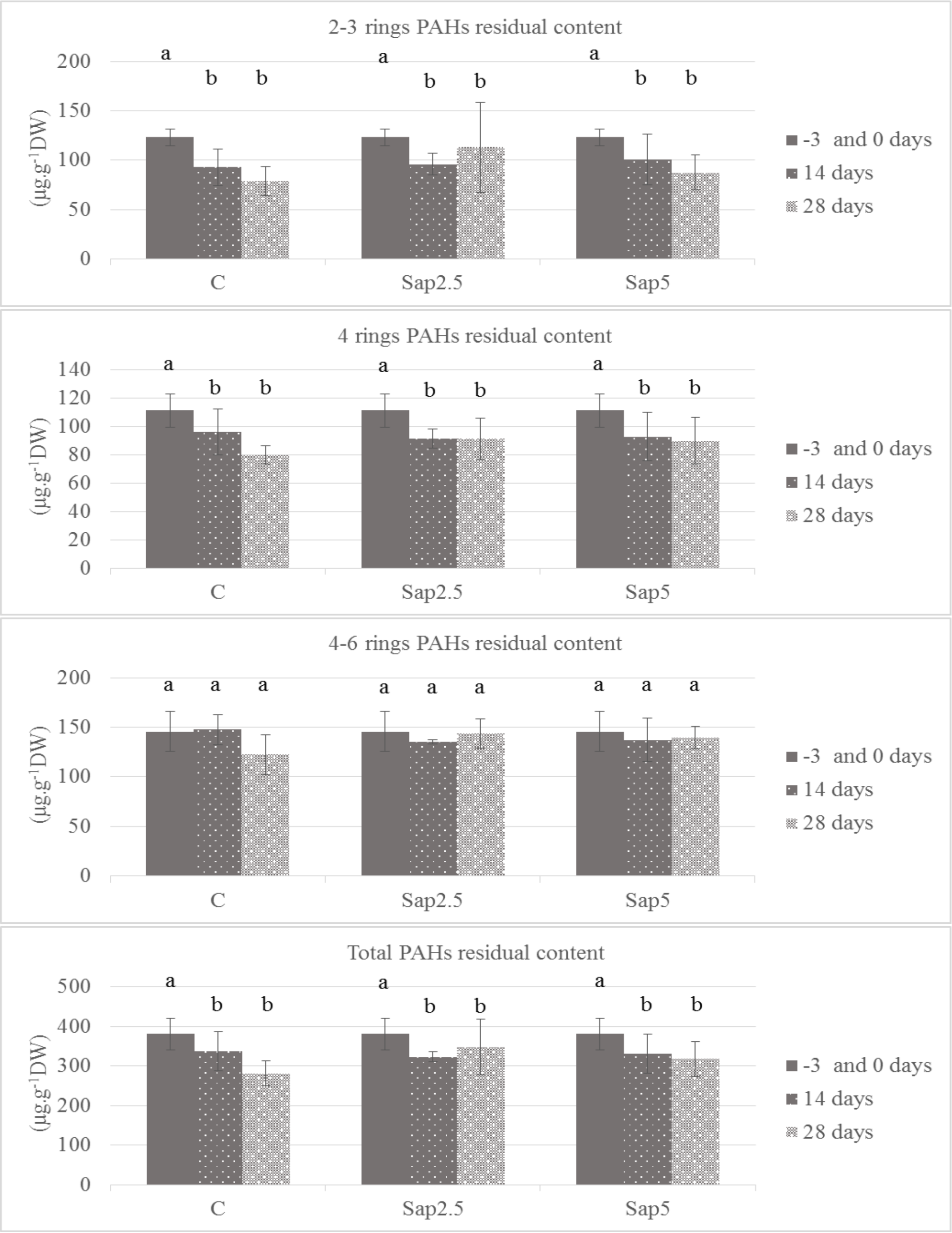


Figure 6. PAHs residual contents of soils treated with commercial saponin (0, 2.5 or 5 mg g^{-1} DW) and after different incubation times. Values are means ± confidence interval (α=5%). Sticks that share the same letter are not significantly different ($p > 0.05$).

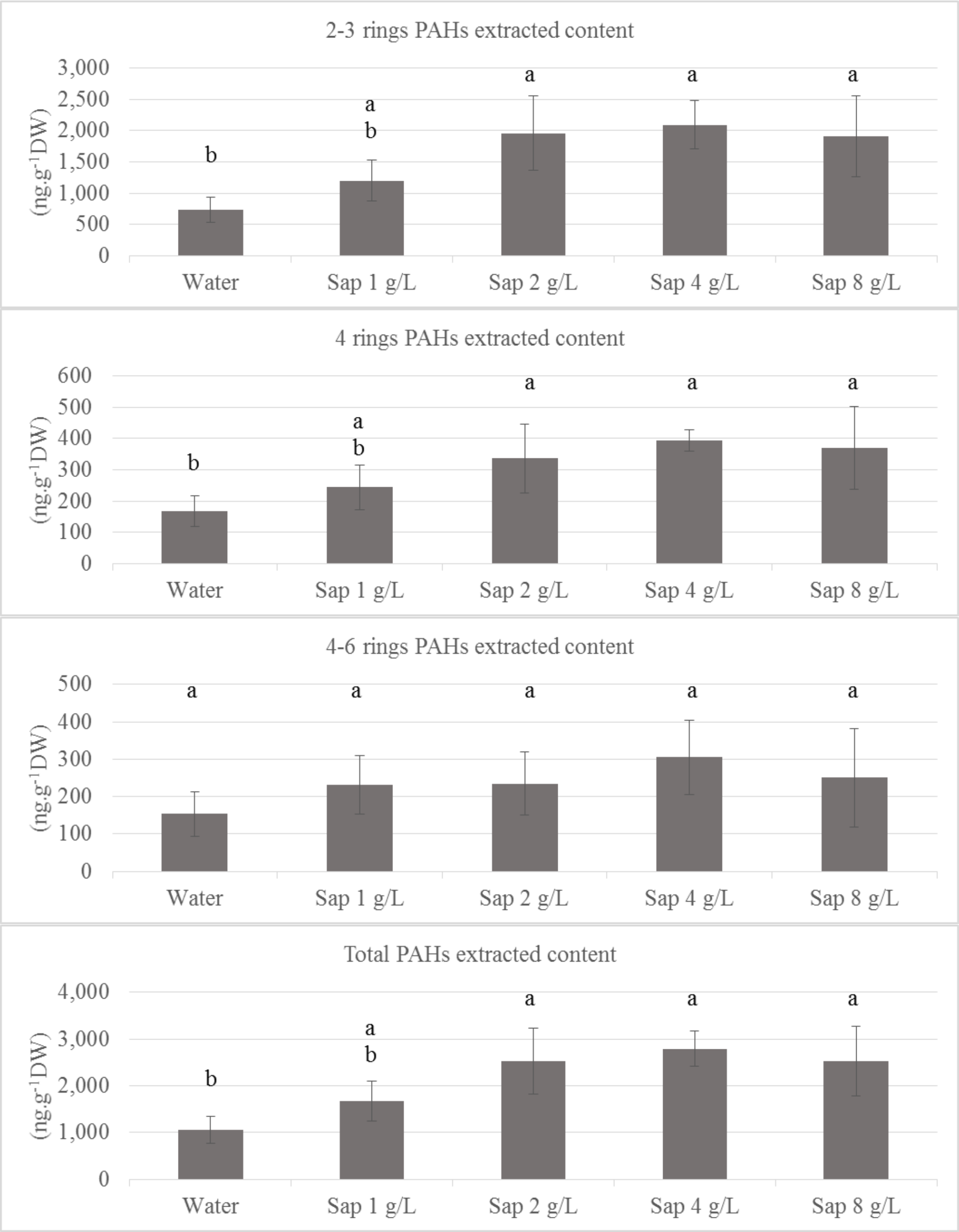


Figure 7. PAHs extracted contents of soils washed with commercial saponin solutions (0, 1, 2, 4 or 8 g L^{-1}). Values are means ± confidence interval (α=5%). Sticks that share the same letter are not significantly different ($p > 0.05$).

Part 2

Investigating the effect of *Medicago sativa* L. and *Trifolium pratense* L. root exudates on PAHs bioremediation in aged-contaminated soils

1. Foreword

The data presented in this section were acquired during the testing of *Medicago sativa* L. and *Trifolium pratense* L. raw root exudates. As explained previously, raw root exudates were harvested from *Medicago sativa* L. and *Trifolium pratense* L. plants grown in hydroponic conditions. Exudates were then used in an incubation experiment.

The experimental aged-contaminated soil was the same as exposed in Part 1. For the experiment, raw exudates (from either *M. sativa* L. or *T. pratense* L.) were amended to soil samples (0 and 5 mg g^{-1} DW). CO_2 emissions were monitored throughout the incubation (1, 3, 7, 14, 21, and 28 days), soil dehydrogenase activities were measured after each incubation period (14 or 28 days), and PAHs residual contents were also acquired using an ISO norm (ISO 13877:1998).

As a reminder, a protocol had to be adapted to the specific experimental soil in order to measure PAHs bioaccessible contents. Since the experimental soil is the same as the one exposed in Part 1, and since bioaccessibility data was not exposed nor published in the article described in Part 1, the complete process of the adaptation of a PAHs bioaccessibility measurement protocol will be exposed hereafter.

All datasets (i.e. CO_2 emissions, dehydrogenase activities, PAHs residual contents and PAHs bioaccessible contents) were discussed in a publication (Davin *et al.,* 2019). The content of this publication is exposed hereafter.

Reference

Davin, M., Starren, A., Marit, E., Lefébure, K., Fauconnier, M.-L., Colinet, G., 2019. Investigating the Effect of *Medicago sativa* L. and *Trifolium pratense* L. Root Exudates on PAHs Bioremediation in an Aged-Contaminated Soil. *Water, Air and Soil Pollution,* 230. https://doi.org/10.1007/s11270-019-4341-4

2. Abstract

Polycyclic aromatic hydrocarbons (PAH) are persistent organic compounds of major concern that accumulate in the environment, especially soils, and require remediation. Researches to develop bioremediation and phytoremediation (alternative eco-friendly technologies) are being conducted. First a bioaccessibility measurement protocol was adapted to a brownfield soil using Tenax® beads in order to compare PAHs bioaccessibility in soil samples. PAHs desorption kinetics were established, described by a site distribution model, and a common extraction time was calculated (48 h). Second the role of two *Fabaceae* (*Medicago sativa* L. or *Trifolium pratense* L.) root exudates in enhancing PAHs bioaccessibility and biodegradation in the studied soil was evaluated during microcosms' experiments (28°C). The CO_2 emissions were significantly higher in presence of *T. pratense* exudates; the dehydrogenase activities showed improvements of the soil microbial activity in presence of two types of root exudates compared to untreated soil samples; the PAHs residual contents decreased more in untreated samples than in the presence of *T. pratense* exudates; and *M. sativa* exudates lowered PAHs bioaccessibility but not residual contents.

3. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are composed of two or more condensed aromatic rings and are usually classified in three main categories: light PAHs of three rings or less, intermediate PAHs of four rings and heavy PAHs of four rings or more (INERIS 2005). These ubiquitous organic compounds are naturally brought into the environment through diagenetic, petrogenetic or pyrolytic processes, but the major source remains incomplete combustions of natural (i.e. volcanic eruptions), and mostly anthropogenic origin such as industrial manufacturing, fuel combustions, or waste disposal. PAHs become more hydrophobic as the number of aromatic cycles raises. Therefore once emitted in the environment, PAHs tend to sorb to solid particles, which renders them less susceptible to biotic and abiotic degradation, and therefore more persistent (Yu *et al.,* 2018). PAHs health-concerning properties are real threats towards ecosystems and motivate the need to develop remediation strategies and control tools.

Over the last decades, the interest in the use of environmental friendly and cost-effective soil remediation techniques has largely increased (Alegbeleye *et al.,* 2017). The use of living microorganisms or plants to dissipate soil pollution is often summarized as *bioremediation* and *phytoremediation* technologies, respectively (Ouvrard *et al.,* 2013). However those techniques can hardly be considered separately as microorganisms and plants closely interact at the soil's solid, liquid and gaseous interfaces. It is indeed now well-acknowledged that plant roots create favorable conditions for microorganisms in their immediate proximity (2 mm), which is named the *rhizospheric effect* (Martin *et al.,* 2014), but also that plant-microbe associations can be beneficial to the plants (Uroz *et al.,* 2019).

Besides favoring the microbial community, studies have shown that the presence of plants also improved PAHs dissipation in contaminated soil. This includes members of the *Fabaceae* family (Wei and Pan, 2010; Hamdi et al. 2012; Alves *et al.,* 2018). *Fabaceae* are good candidates for phytoremediation on brownfield soils because they are capable of colonizing hydrocarbon contaminated soils which often present very high carbon-nitrogen ratio (Hall et al. 2011). However the mechanisms through which plants enhance PAHs biodegradation in soil (i.e. rhizodegradation) are not yet fully understood.

Biodegradation processes are balanced by two major phenomena: (i) the mass transfer of a compound to a microbial cell and (ii) the uptake and metabolization of this compound by the living cell. The pollutant intrinsic physico-chemical properties (i.e. aqueous solubility, hydrophobicity, and molecular structure), along with environmental factors (such as content and nature of organic matter or clay minerals in soil), will influence the compound concentrations in the aqueous phase and thus their accessibility to degrading agents (microorganisms and their enzymes). Other factors such as pH, salinity, temperature, water content, mineral nutrients, redox potential, and water-dissolved oxygen will provide conditions more or less favourable to the microbial activity (Haritash and Kaushik 2009).

When it comes to rhizodegradation the general explanation found in the literature suggests that the enhanced dissipation of PAHs is caused by the rhizospheric effect, which itself is a combination of several physical and chemical phenomena: (i) increased contact between soil and microorganisms (Ouvrard *et al.,* 2014), (ii) soil aeration, and (iii) the release of exudates by plant roots which provides the microbiota with easily accessible carbon sources and thus increases microbial communities (Alagić et al. 2015).

Indeed the majority of root exudates are composed of organic acids, sugars and amino acids. But studies about secondary plant metabolites in general have shown a large diversity of compounds that are released in the environment, some of which exhibit tensioactive (or surfactant) properties due to an amphiphilic nature. Such compounds are very often heterosides, (a hydrophobic skeleton of steroidal or triterpenoidal nature coupled to a glycose (hydrophilic) moiety), and commonly referred to as saponins. Such compounds have been detected in members of the *Fabaceae* family (Vincken et al. 2007; Kregiel et al. 2017). Surfactants can place themselves at the interface between a hydrophobic and a hydrophilic phase and have been the subject of soil remediation studies, either in "washing technologies" (Von Lau et al. 2014) or to enhance mass transfer of contaminants towards degrading microorganisms (Kobayashi et al. 2012).

Based on this literature, a study was designed to determine the role of root exudates from two *Fabaceae* (*Medicago sativa* L. or *Trifolium pratense* L.) in enhancing PAHs bioaccessibility as part of the *rhizospheric effect*.

Hydrophobic organic compounds (HOCs) bioavailability/bioaccessibility has been intensively discussed (Ehlers and Luthy 2003; Semple et al. 2004) and will not be reminded here. However, the scientific community agrees that the fraction of a contamination that is the most likely to be degraded by the soil microbiota will be accessed in the aqueous phase. That fraction is named "bioaccessible", according to Semple et al. (2003) (i.e. "the compound that is available to cross an organism's cellular membrane from the environment, if the organism has access to the chemical"). Therefore analytical developments have been oriented to give the closest representation of the HOCs fraction that is bioaccessible to microorganisms in order to evaluate the potential for bioremediation of a given soil (Semple et al. 2003).

Cornelissen et al. (1997) developed a solid-phase extraction technique using Tenax® beads that mimic the interaction between the contaminants and the microbiota in the aqueous phase, if all the bioaccessible contaminants were degraded by these organisms. The determination of the accessible fraction of a contamination is however directly related to the time of contact between the microbial surrogate (the Tenax® beads) and the aqueous phase of a soil, and therefore desorption kinetics of a compound in a contaminated soil must be established to determine a minimal time of contact. As this time of extraction must be representative of a compound's bioaccessibility, it also should be economically affordable and cost-effective if the analytical method is to be applied routinely (for example to monitor the bioaccessibility of a pollutant in a soil, whether a specific treatment is applied or not).

Several models have been used by searchers to describe HOCs, and more specifically PAHs, desorption kinetics from soils. It is generally admitted that PAHs desorption occurs in several stages (Richardson and Aitken 2011). In an attempt to simplify descriptions, the compartment model is often used to reduce the phenomenon to a few representative stages described by first-order kinetics. The first stage is the rapid release of the most accessible fraction (F_{rap}) of the PAHs and is assimilated to the fraction that could be degraded by microorganisms. Another model, the site distribution model (first suggested by Connaughton *et al.,* 1993) is based on a gamma distribution of rate coefficients, and considers the system as a continuum of compartments. While the use of this model does not allow to properly quantify rapidly and slowly desorbing fractions, it is probably more representative of the actual processes than the compartment model.

To evaluate the role of root exudates on the PAHs bioaccessible fraction, the first step of the present study was to adapt a bioaccessibility measurement protocol to the studied contaminated soil. Therefore, desorption kinetics of PAHs in the studied soil were determined and described using a model. Afterwards, a common and cost-effective Tenax® beads extraction time was established as a comparison basis for PAHs bioaccessibility assessments. In a second time, contaminated soil was incubated in presence of plant-root exudates in an attempt to enhance PAHs bioaccessibility. Two types of exudates and two incubation periods were tested while several parameters were examined: (i) the carbon dioxide emission was monitored during the incubation process to assess for microbial activity; (ii) dehydrogenase activity was determined at the end of each incubation period as an indicator of the soil microbial activity; (iii) the residual PAHs contents and (iv) the bioaccessible PAHs were determined on soil samples after each incubation period to evaluate the impact of plant-root exudates on PAHs dissipation and bioaccessibility.

4. Materials and methods

4.1. Soil material

The experimental aged-contaminated soil has already been described in a former study (Davin *et al.*, 2018) but its characteristics will be reminded hereunder. The soil was sampled from a brownfield in Saint-Ghislain (Belgium) in a former coking plant and has been exposed for 70 years to petroleum hydrocarbons, PAHs, cyanides and trace elements. Particle size distribution (81.1 % sand, 10.7 % silt, 8.2 % clay) identified the soil as loamy sand, pH was 6.7, total organic carbon was 9.44 ± 0.22 % (W/W), and total nitrogen content was 0.16 ± 0.02 % (W/W). Soil was sampled, allowed to dry at ambient air, sieved through a 2-mm sieve and stored in sealed boxes until further use. Before the experiments, the contents of 15 PAHs were determined to range from 2.9 ± 0.1 µg g^{-1} DW to 65.9 ± 7.1 µg g^{-1} DW (initial individual concentrations are in online resource 1). The studied PAHs are Acenaphtene (Ace), Anthracene (Anthr), Benzo[a]anthracene (BaA), Benzo[a]pyrene (BaP), Benzo[b]fluoranthene (BbF), Benzo[ghi]perylene (BghiP), Benzo[k]fluoranthene (BkF), Chrysene (Chrys), Dibenzo[ah]anthracene (DBahA), Fluoranthene (F), Fluorene (Fle), Indeno[123-c,d]pyrene (IcdP), Naphthalene (N), Phenanthrene (Phen), and Pyrene (Pyr).

4.2. Plant root exudates: production and characterization

Plant root exudates production was inspired by Louvel (2010). Seeds of *Medicago sativa* L. and of *Trifolium pratense* L. were purchased from Ecosem and presented a germination rate of over 95%. After surface sterilization in a 6% (w/v) solution of hydrogen peroxide for ten minutes, plants were grown on hydroponic floating devices; using Hoagland's nutritive solution (Hoagland and Arnon 1950). Air-blowers allowed proper oxygenation for the roots and plants were kept in a greenhouse where lamps assured 12h of light per day when necessary. Once a week, root-parts were rinsed of the nutritive solution and placed in 1 litre of distilled water for 5 hours. The aqueous solution was filtered on paper filter (11 µm), frozen and lyophilized. Remaining dry exudates were homogenized and stored at - 20°C until further use. All exudates were pooled together by plant type. The total organic carbon and the total nitrogen contents were respectively 11.37±0.22% and 0.868±0.016% (w/w) for *Medicago sativa* exudates (E_MS), and 10.46±0.22% and 0.984±0.016% (w/w) for *Trifolium pratense* exudates (E_TP).

4.3. PAHs desorption kinetics

Desorption kinetics was measured five times according to a method adapted from Cornelissen et al. (1997) and Barnier et al. (2014). Briefly, 2.0 g of soil were weighed into glass centrifuge tubes. 50 mL of 0.01 M $CaCl_2$ and 0.003 M NaN_3 were added as biocides along with 0.5 g of Tenax® beads (60-80 mesh). The tubes were shaken for 1, 2, 4, 8, 16, 24, 48, 72 or 96 hours on a rotary agitator (40 cycles min^{-1}). Tubes were then centrifuged (10 min; 2000 x g) to separate the soil from the Tenax® beads. The floating beads were separated by filtration on a Buchner vacuum device and air dried. Sorbed PAHs were recovered from Tenax® by a 60 min sonication with 20 mL of a 50:50 (V/V) n-hexane: acetone mixture, repeated three times. The organic phase was evaporated with a rotative evaporation device, and replaced with acetonitrile. The final acetonitrile extract was weighed for volume determination and analysed for PAHs. Each PAH amount extracted by Tenax® beads was then used to calculate the remaining sorbed fraction in soil as follows

$$\frac{S_t}{S_0} = \frac{C_{tot_{in}} - C_{ext_t}}{C_{tot_{in}}} \qquad (1)$$

where $C_{tot\ in}$ is the total initial PAH concentration in the soil [µg g^{-1} DW]; $C_{ext\ t}$ is the amount of PAH adsorbed by Tenax ® beads after t hours of extraction [µg g^{-1} DW]; S_t is the sorbed fraction of compound remaining after t hours of extraction; and S_0 is the initial sorbed fraction, assumed to be the total initial PAH concentration.

4.4. Incubation experiments

Incubation experiments were conducted in microcosms according to AFNOR XP U44-163. Briefly, 15 g of dry soil were pre-incubated for 3 days at 80 % of water holding capacity. Once amendments were added to samples (day 0 of incubation), two vessels were placed next to each sample in a sealed jar. One vessel was filled with distilled water to prevent soil desiccation and one was filled with NaOH solution to control carbon dioxide emission. Exudates were added to soil samples in order to reach 5 mg g^{-1} DW, for both plant types. Untreated soil served as control and two incubation periods (14 and 28 days starting at the addition of exudates) were tested. All modalities were repeated four times for a total of 24 samples. All jars were sealed and incubated at 28°C, in the dark. At the end of the incubation period, soils were sacrificed for dry weight, dehydrogenase activity and PAHs measurements (residual and bioaccessible) concentrations. Results related to soil samples with 5 mg g^{-1} DW of *Medicago sativa* L. or *Trifolium pratense* L. exudates are named E_MS and E_TP, respectively. Results related to control samples are named C.

4.5. *Chemical analyses*

Dry weight determination.

Soil samples dry weight determination was based on ISO 11465:1993 cor 1994.

Total nitrogen content.

Total nitrogen determination was based on ISO 11261:1995.

Total organic carbon.

Total organic carbon determination was based on ISO 14235:1998.

Carbon dioxide emission.

Carbon dioxide emission was monitored for each soil sample throughout the whole incubation following AFNOR XP U44-163.

Dehydrogenase activity.

Dehydrogenase activity was measured for each soil sample after the incubation following a method described by Shaw and Burns (2005).

Bioaccessible PAHs determination in soil samples.

Bioaccessible PAHs determination in soil samples was realised on fresh soil samples as described in the PAHs desorption kinetics section, except the samples were agitated for 48 hours in the presence of the Tenax® beads (see the PAHs desorption parameters paragraph of the results section for time choice).

Total PAHs determination in soil samples.

Total PAHs extraction in soil samples was based on ISO 13877:1998. The final acetonitrile extract was analysed for PAHs.

PAHs analysis.

PAHs were analysed in acetonitrile extracts of desorption kinetics, bioaccessible and residual samples based on ISO 13877:1998.

Models and statistics.

R 3.4.3 was used to generate PAHs desorption models. The Levenberg-Marquardt algorithm was used to minimize squared residuals between experimental and calculated values for each or the four tested models (Table 5) (Prague et al. 2012). A model was selected for each PAH using the Bayesian information criterion (BIC) which estimates the relative information of a model as follows

$$BIC = k.\ln(n) - 2.ln(L) \tag{2}$$

where k is the number of parameters of a model, n is the number of data points and L is the maximized value of a likelihood function. R function is BIC(model_iner2).

All statistical analyses related to the incubation experiment were carried out using Minitab 17.0. Equality of variances were verified according to Levene’s test, data were analysed by general linear model or one-way analysis of variance and mean values were compared by Tukey’s test at the 5 % confidence level.

Table 5. Desorption theoretical models and their characteristics.

Desorption model	**Equation**	**Number of parameters**
First-order model	$\frac{S_t}{S_0} = e^{-kt}$	1
First-order two-compartment model	$\frac{S_t}{S_0} = F_{rap} * e^{-k_{rap}t} + F_{slow} * e^{-k_{slow}t}$ $F_{rap} + F_{slow} = 1$	4
First-order three-compartment model	$\frac{S_t}{S_0} = F_{rap} * e^{-k_{rap}t} + F_{int} * e^{-k_{int}t} + F_{slow} * e^{-k_{slow}t}$ $F_{rap} + F_{int} + F_{slow} = 1$	6
Site distribution model	$\frac{S_t}{S_0} = \left(\frac{\beta}{\beta + t}\right)^{\alpha}$	2

5. Results and discussion

5.1. *Assessing PAHs bioaccessibility*

Modelling PAHs desorption kinetics.

Soil samples were extracted for increasing time steps in the presence of Tenax® beads and the recovered PAHs amounts were used to calculate remaining sorbed fractions for each extraction time according to equation (1) (data is available in Supplementary table 2). Then modelling was used to describe desorption kinetics (Figure 8). BIC values were calculated using R for each tested model and are available in Supplementary table 3. These values have no meaning by themselves and can only be used to compare models generated from a same data set. The smallest BIC value indicates the model that better represents the data set and was obtained by the site distribution model for all compounds except for the heaviest PAHs (DBahA, BghiP, IcdP) for which it was obtained by the first-order three-compartment model. These three compounds showed BIC-value differences of four to six units with the second-best model, which in each case was the site distribution model. According to Kass and Raftery (1995) this range of difference of BIC value between models is positive, but not strong. Therefore, to homogenize the description of desorption kinetics, the site distribution model was chosen for all compounds (Figure 8).

PAHs desorption parameters.

Desorption models were used to determine a minimal extraction time (t_{ex}) for bioaccessibility measurement of each PAH. This t_{ex} should represent the time for the most accessible fraction to equilibrate with Tenax® beads. Therefore, t_{ex} values were calculated as the time for which the slope to the desorption model closes down to zero. Given the asymptotic nature of the models, the slope limit was arbitrarily set to 10^{-3} and successive approximations were made according to the following equation

$$\frac{y_{t_{ex}-24}-y_{t_{ex}}}{24} \leq 0.001 \qquad (3)$$

where y is the calculated value of a PAH site distribution equation at different times; and t_{ex} is the extraction time [h].

Calculated t_{ex} values and site distribution models parameters (alpha and beta) are presented in Table 6. Alpha values range from $6.88.10^{-3}$ to $1.14.10^{-2}$, beta values range from $8.98.10^{-4}$ h to 1.34 h, and calculated extraction times are either 24 h (for the lightest PAHs) or 48 h. Thus a common 48 h extraction time was used to determine PAHs bioaccessible contents in the incubation experiment. Let us stress here that the “bioaccessible contents” that will be discussed further down actually are “contents that are extracted after 48 h of presence of Tenax® beads.”

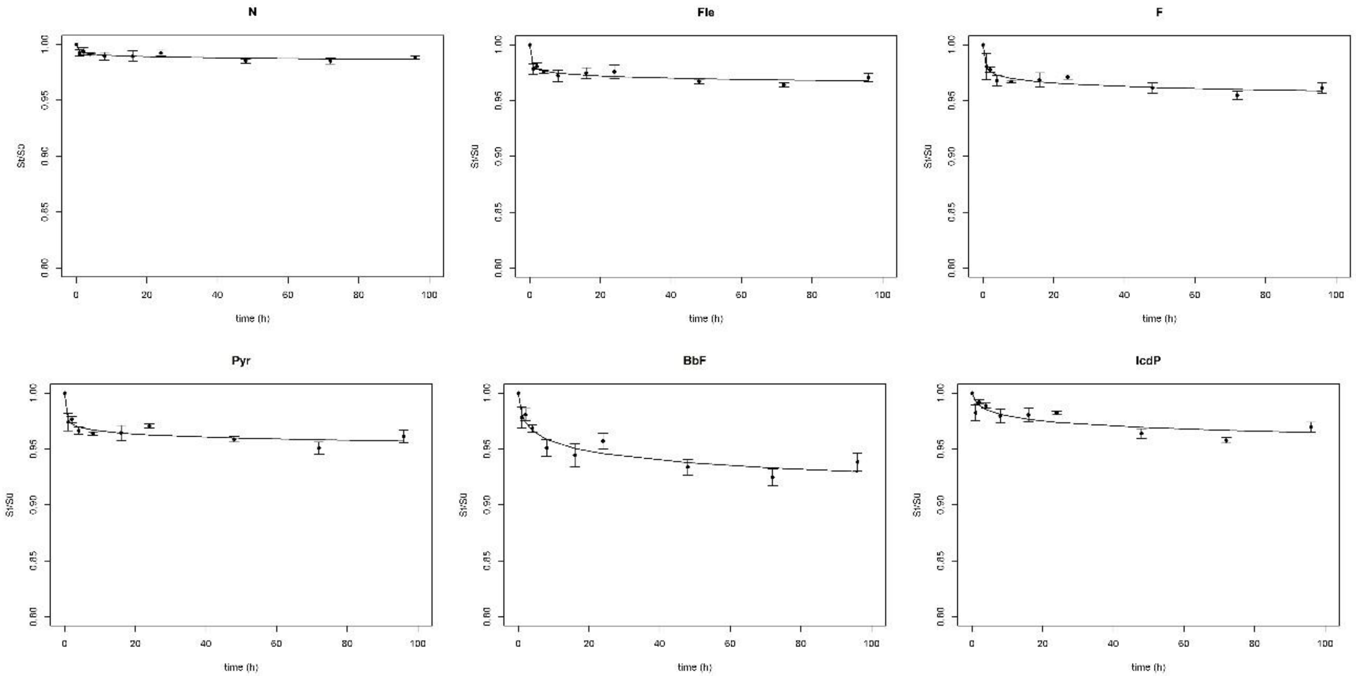


Figure 8. Examples of PAHs desorption kinetics using Tenax®. S_t/S_0 is the remaining sorbed fraction according to extraction time. Dots are data means ± confidence interval (n=5), lines are fitted site distribution models.

Table 6. Fitted parameters of the site distribution model for the different PAHs and t_{ex} values calculated according to equation (3).

PAHs*	**β (h)**	**α (-)**	**t_{ex} (h)**
N	$1.54.10^{-2}$	$1.53.10^{-3}$	24
Ace	$6.82.10^{-4}$	$1.22.10^{-3}$	24
Fle	$8.98.10^{-4}$	$2.83.10^{-3}$	24
Phen	$2.00.10^{-3}$	$3.91.10^{-3}$	48
Anthr	$9.30.10^{-3}$	$1.27.10^{-2}$	48
F	$1.05.10^{-2}$	$4.61.10^{-3}$	48
Pyr	$2.43.10^{-3}$	$4.14.10^{-3}$	48
BaA	$1.02.10^{-1}$	$1.14.10^{-2}$	48
Chrys	$1.24.10^{-1}$	$1.53.10^{-2}$	48
BbF	$2.78.10^{-1}$	$1.24.10^{-2}$	48
BkF	$6.03.10^{-1}$	$1.45.10^{-2}$	48
BaP	$5.54.10^{-1}$	$1.12.10^{-2}$	48
DBahA	$1.34.10^{0}$	$1.15.10^{-2}$	48
BghiP	$1.95.10^{-1}$	$4.66.10^{-3}$	48
IcdP	$5.29.10^{-1}$	$6.88.10^{-3}$	48

*PAHs are sorted by increasing molecular weight

5.2. PAHs bioremediation in presence of root exudates

Respiration curves and dehydrogenase activities.

Figure 9 presents CO_2 emissions of (un)treated soil samples throughout incubation in microcosms. Statistical analysis was performed after $\log_{10}$ transformation.

E_TP soil samples exhibit significantly higher cumulated CO_2 emissions than C and E_MS samples after 7, 21, and 28 days of incubation (p=0.000). E_MS however is never significantly different from C samples. Assuming that all the amendments added to E_TP and E_MS samples had been completely mineralized, CO_2 emissions would be of respectively 1.92 ± 0.04 and 2.08 ± 0.04 mg CO_2 g^{-1} DW. In the case of E_MS samples, the observed emission is lower than the calculated emission, but in the case of E_TP samples it is higher, suggesting that TP exudates influence CO_2 emissions to a greater extent than their own degradation, and also that MS exudates were not entirely mineralized.

Figure 10 shows (un)treated soil samples dehydrogenase activities before and after incubation. There is a significant interaction between time and treatment. C samples activities decrease throughout the incubation and are significantly lower after 28 days of incubation than at the beginning of the incubation. E_TP and E_MS samples, however, show increases after 14 days (respectively + 134% and + 99.5%) before lowering back during the last two weeks of incubation. Being an indicator of soil general health (Das and Varma 2011), the raise in this enzyme activity suggests that the amended exudates have no toxic effect towards the soil microbiota.

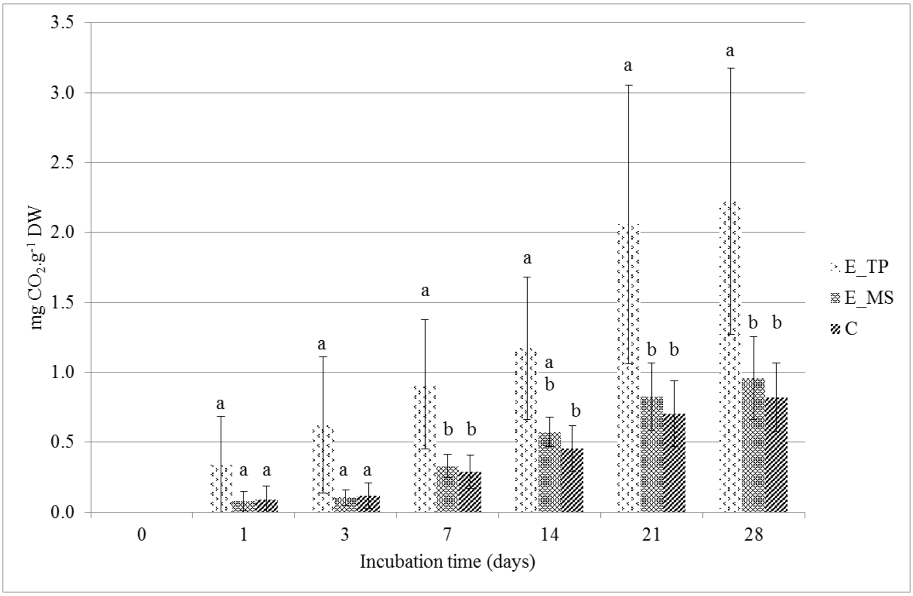


Figure 9. CO_2 cumulated emissions during the incubation of soils treated with *Medicago sativa* (E_MS) or *Trifolium pratense* (E_TP) plant root exudates compared to untreated samples (C). Values are means ± confidence interval (α=5%). Within each time group, treatments sharing the same letter are not significantly different ($p > 0.05$).

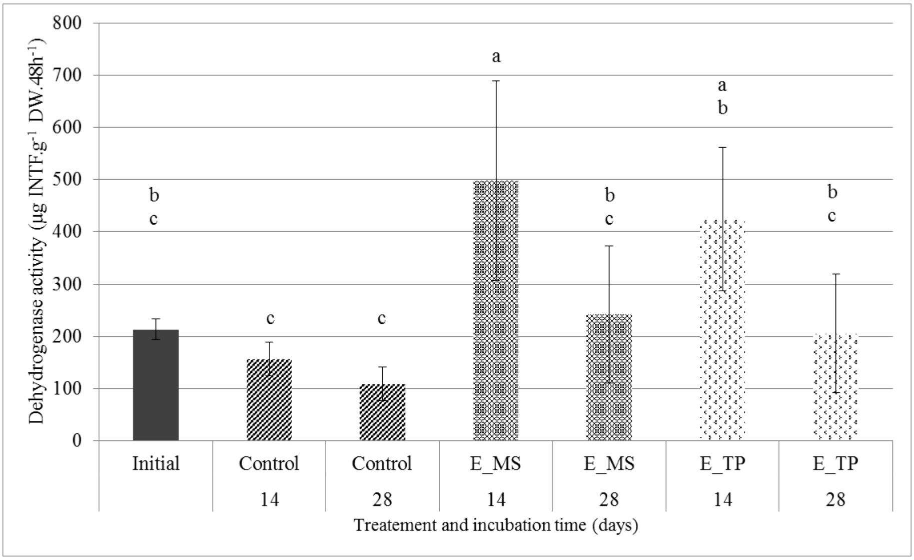


Figure 10. Dehydrogenase activities of soils treated with *Medicago sativa* (E_MS) or *Trifolium pratense* (E_TP) plant root exudates, compared to untreated samples (C) after different incubation periods. Values are means ± confidence interval (α=5%). There is a significant interaction between time and treatment. Sticks that share the same letter are not significantly different ($p > 0.05$).

PAHs residual and bioaccessible contents.

Figure 11 and Figure 12 respectively show (un)treated soil samples residual and bioaccessible PAHs contents before and after incubation.

For both sets of results, PAHs contents were grouped to provide better information: 2-3 rings (N, Ace, Fle, Phen, and Anthr); intermediate 4 rings (F and Pyr); 4-6 rings (BaA, Chrys, BbF, BkF, BaP, DBahA, BghiP, and IcdP); and total PAHs (N to IcdP). Statistical analyses on bioaccessible contents were performed after $\log_{10}$ transformation. Significant differences appear between the bioaccessible contents measured on the untreated soil to establish desorption kinetics and the bioaccessible contents measured after 3 days of pre-incubation (respectively named “-3 days” and “0 days” in Figure 12). After this pre-incubation period, the bioaccessible contents are respectively three (2-3 rings PAHs), four (intermediate 4 rings PAHs), two (4-6 rings PAHs), and three (total PAHs) fold the ones measured initially in desorption kinetics.

Statistical analyses on both residual and bioaccessible contents show no interaction between time and treatment. Different behaviours appear within each treatment. (i) The residual content of 2-3 rings PAHs is significantly lower ($p<0.05$) for E_TP and E_MS samples and very significantly lower ($p<0.01$) for C samples after 14 days of incubation whilst the bioaccessible content of 2-3 rings PAHs is highly significantly lower ($p=0.000$) after 14 days of incubation for each treatment. PAHs could have been dissipated from the soil by biotic (such as biodegradation) or abiotic processes (such as volatilization, which would not come as a surprise for a volatile compound such as naphthalene with a vapour pressure of 10.5 Pa at 25°C). Regardless, this means the less sorbed fraction of light PAHs was eliminated from the soil, and was not replaced. So the remaining PAHs are more or less strongly sorbed to the soil and for this group of PAHs, the addition of TP or MS exudates does not enhance dissipation compared to untreated samples.

(ii) The residual content of intermediate 4 rings PAHs is significantly lower for E_TP samples after 14 days and for C samples after 28 days of incubation, whilst there is no significant lowering of this PAHs group in E_MS samples after 28 days. On the other hand, the bioaccessible sum of intermediate 4 rings PAHs is highly significantly lower ($p=0.000$) after 14 days of incubation for each treatment. The fact that this group of PAHs dissipates faster in E_TP than in C samples is probably caused by the addition of TP exudates that provided a more easily available source of carbon for the soil microbiota (Louvel, 2010) and boosted its activity, allowing it to start degrading PAHs faster. In the case of E_MS samples though, the fact that this group of PAHs bioaccessibility lowers significantly whilst their residual content remains statistically unchanged suggests that MS exudates might be preventing PAHs to be dissipated by influencing their bioaccessibility.

(iii) The residual content of 4-6 rings PAHs does not significantly lower after 28 days of incubation for any treatment. As for the bioaccessible content of 4-6 rings PAHs, after being enhanced by the pre-incubation process, it lowers back towards the initial (-3 days) level of bioaccessibility for each treatment. This suggests that the stirring and addition of water might have enhanced those highly hydrophobic PAHs bioaccessibility for a short time before PAHs sorbed back to soil particles, either because they could or were not yet dissipated.

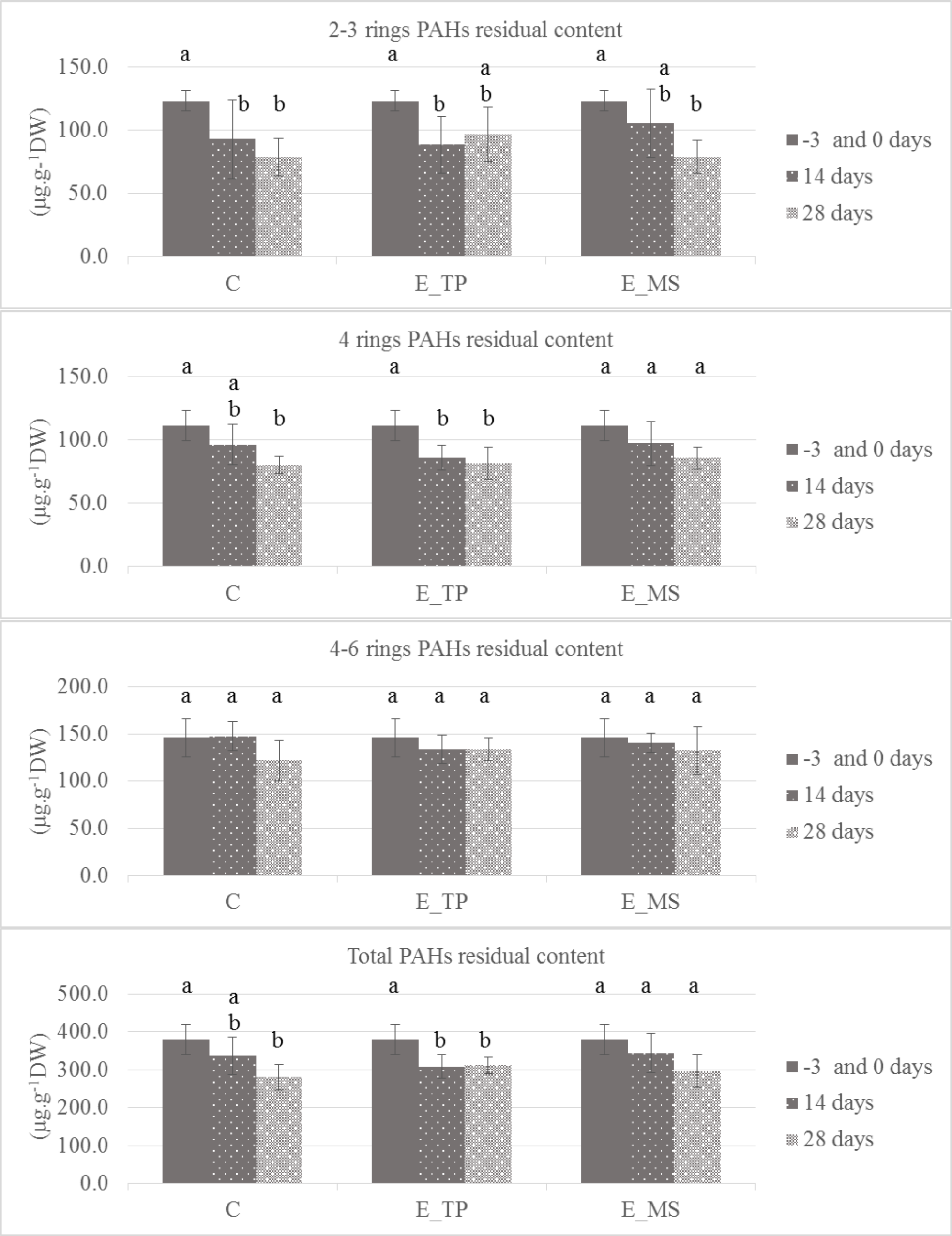


Figure 11. PAH residual contents of soils treated with *Medicago sativa* (E_MS) or *Trifolium pratense* (E_TP) plant root exudates, compared to untreated samples (C) after different incubation periods. Values are means ± confidence interval (α=5%). Within each treatment group, sticks that share the same letter are not significantly different ($p > 0.05$).

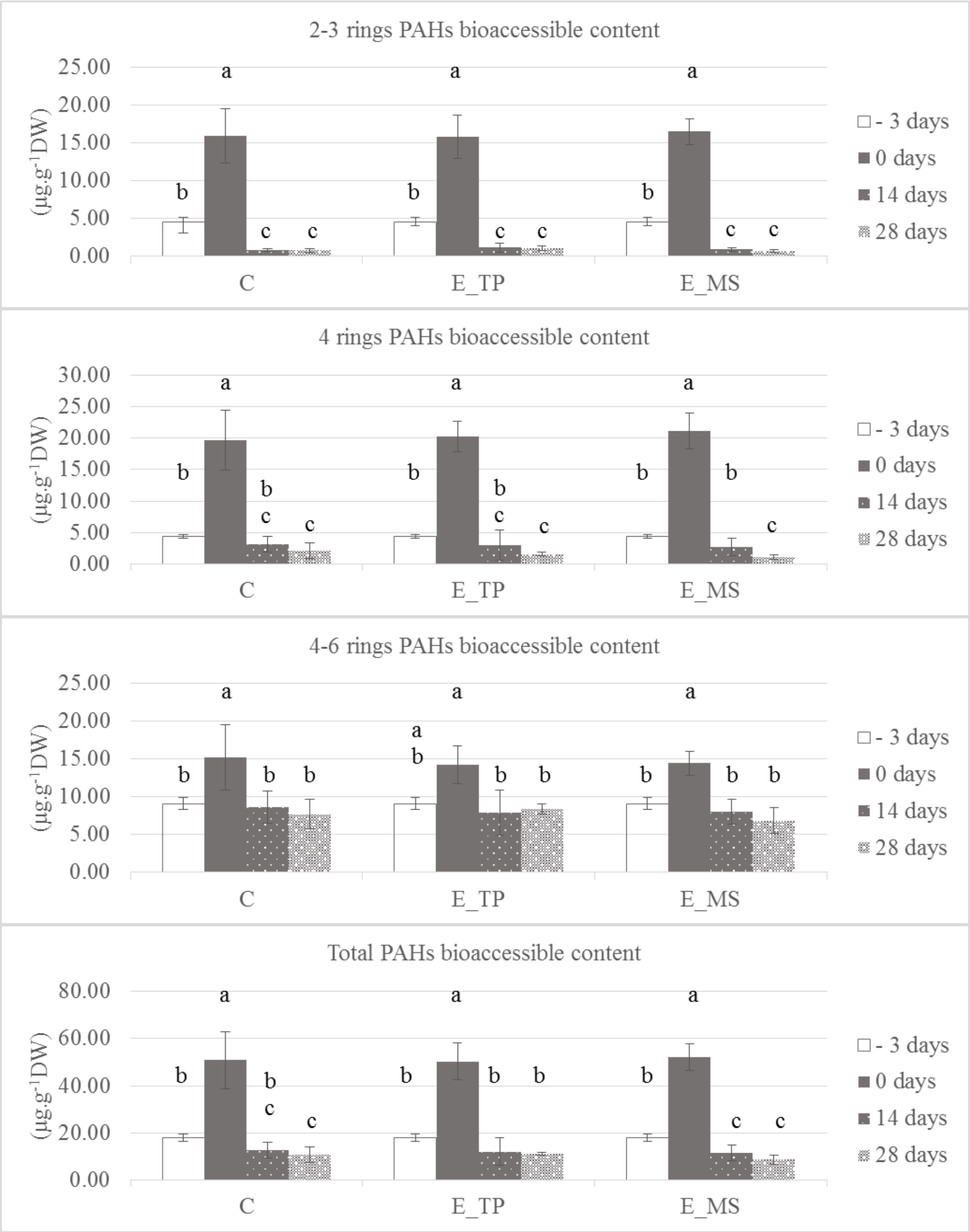


Figure 12. PAH bioaccessible contents of soils treated with *Medicago sativa* (E_MS) or *Trifolium pratense* (E_TP) plant root exudates, compared to untreated samples (C) after different incubation periods. Data before and after the pre-incubation period are respectively named "-3 days" and "0 days". Values are means ± confidence interval (α=5%). Within each treatment group, sticks that share the same letter are not significantly different ($p > 0.05$).

(iv) The global residual and bioaccessible contents of all PAHs confirm some previously made observations. The total residual PAHs content is significantly lower ($p<0.05$) after 14 days for E_TP samples and after 28 days for C samples but is not different after 28 days for E_MS samples. On the other hand the total bioaccessible PAHs content is highly significantly lower ($p=0.000$) than prior the incubation after 14 days for E_MS samples and after 28 days for C samples. Here again this suggests that TP exudates enhanced soil microbial activity, allowing PAHs dissipation to start faster than in C samples. This hypothesis is supported by the significantly more important CO_2 emissions observed in E_TP samples (Figure 9) and the higher dehydrogenase activity (showing soil microbiota enhanced activity) in Figure 10. But this easily available carbon source was also probably favoured to PAHs throughout the incubation (Cébron et al. 2011), which could explain why C and E_TP total residual contents are statistically not different after 28 days of incubation. As for MS exudates negatively influencing PAHs dissipation, it is reinforced by the fact that CO_2 emissions in E_MS samples were not different from the ones in C samples, suggesting that MS exudates were not favoured to PAHs as a carbon source but also that there was not much mineralization taking place in the microcosm. Such results are surprising since MS exudates should also constitute an easily accessible source of carbon for the microbiota, and dehydrogenase activities were also enhanced in the presence of MS exudates.

6. Conclusions and perspectives

The objectives of the exposed experiments were to adapt a common and cost-effective Tenax ® beads extraction protocol to an aged-contaminated soil that would serve as a comparison basis for PAHs bioaccessibility measurements; and to evaluate the role of *Medicago sativa* L. and *Trifolium pratense* L. root exudates in enhancing PAHs bioaccessibility and biodegradation in an aged-contaminated soil.

PAHs desorption kinetics were established and described by the site distribution model. The models' parameters helped calculate minimal extraction times for all compounds and a common extraction time was determined (48 h).

The results from the incubation experiment strongly suggest that the global dissipation of PAHs is not enhanced by the presence of *Medicago sativa* L. nor *Trifolium pratense* L. root exudates at least in a relatively short time (28 days) and is equivalent in control samples.

This suggests that humidification, oxygenation and a little heating is enough for the natural microbiota to attenuate the pollution, rendering other treatments pointless. However, the parallel diminution of PAHs bioaccessibility and dehydrogenase activity suggest that dissipation in untreated samples is likely to reach a limit. Indeed in a logic of soil remediation through biodegradation (for which the dissipation must be carried as far as possible and the microbiota must reach the contaminants), the balance between mass transfer and microbial degradation should be maintained (Johnsen et al. 2005). In order to achieve that, bioaccessible contents would have to remain similar until the dissipation is more advanced, and it is not the case here. On the other hand, a diminution of the bioaccessible contents also means the threat to the environment is diminished because the remaining contaminants are more strongly sorbed to soil particles and thus less likely to be accessed by soil organisms through the soil's aqueous phase, which is positive from a risk analysis point-of-view.

The incubation period was a norm-based protocol decision and a longer incubation might have shown different results on the long-term. The increase of dehydrogenase activities in presence of both *Medicago sativa* L. and *Trifolium pratense* L. root exudates show a temporary improvement of soil microbial activity. Therefore, a longer pre-incubation period followed by regular exudates inputs might have allowed the dissipation of bioaccessible PAHs before exudates were added. Maybe such treatment would, in the presence of *Trifolium pratense* L. root exudates, improve soil microbial activity on the long term or eventually influence PAHs bioaccessibility. This would be coherent with the hypothesis that *Trifolium pratense* amendments were preferably used as a carbon source by the soil microbiota throughout the incubation. However it does not explain why PAHs bioaccessibility is globally lowered in presence of *Medicago sativa* exudates whilst the global content is not.

Medicago sativa L. and *Trifolium pratense* L. are both *Fabaceae* species, possess a fibrous root system and are nitrogen-independent due to symbiotic relationships with nitrogen fixating rhizobia (Hall et al. 2011). The similarities would be expected to extend to their root exudates characteristics but evidently differences led to different outcomes on PAHs bioaccessibility and dissipation in soil.

The experiment was initially designed based on the knowledge that *Fabaceae* root exudates produce surface-active compounds and under the hypothesis that they could enhance organic compounds bioaccessibility. However, studies on surfactants also mention that hydrophobic interactions can take place between surfactants and soil particles (Laha et al. 2009), and that partitioning of HOCs into soil-sorbed surfactants could enhance the contaminants sorption to soil. Similar assumptions were made in a previous study aiming to increase PAHs apparent solubility in presence of saponins from *Quillaja saponaria* bark (Davin et al. 2018). The results showed that if the surfactant concentration was too elevated, PAHs solubilisation was less efficient, maybe because PAHs were secluded by saponins micelles or hemimicelles.

The reasons for a diminution of global PAHs bioaccessibility in presence of *Medicago sativa* L. root exudates would have to be investigated through the extraction, characterization and testing of surface-active compounds in exudates (many protocols relying on chromatographic and spectral techniques exist and have been reviewed by Oleszek and Bialy (2006)). If *Medicago sativa* L. exudates turned out to present stabilization properties towards organic contaminants such as PAHs, maybe this type of amendment could be investigated as a secluding agent to slow down a pollution migration, for example.

For now and from a PAHs-remediation point-of-view, the results suggest that *Medicago sativa* L. and *Trifolium pratense* L. root exudates, when added in a single dose, do not enhance PAHs bioaccessibility in the tested soil, and that simple soil moisturizing and incubation, as applied in control samples, leads to identical PAHs dissipation, at least on the short-term. However, it would be of great interest to evaluate whether the growth of whole *Medicago sativa* L. or *Trifolium pratense* L. plants on contaminated soils affects PAHs bioaccessibility and dissipation in similar ways, given that root exudates are released at different, continuous rates *in situ*.

7. References

AFNOR XP U44-163 Amendements organiques et supports de culture - Caractérisation de la matière organique par la minéralisation potentielle du carbone et de l'azote.

Alagić, S., Maluckov, B.S., & Radojičić, V.B., 2015. How can plants manage polycyclic aromatic hydrocarbons? May these effects represent a useful tool for an effective soil remediation? A review. *Clean Technol. Environ. Policy* 17(3), 597–614.

Alegbeleye, O.O., Opeolu, B.O., & Jackson, V.A., 2017. Polycyclic Aromatic Hydrocarbons: A Critical Review of Environmental Occurrence and Bioremediation. *Environ. Manage.* 60(4), 758–783.

Alves, W.S., Manoel, E.A., Santos, N.S., Nunes, R.O., Domiciano, G.S., Soares, M.R., 2018. Phytoremediation of polycyclic aromatic hydrocarbons (PAH) by cv. Crioula: A Brazilian alfalfa cultivar. *Int. J. Phytoremediation* 20(8) 747-755.

Barnier, C., Ouvrard, S., Robin, C., Morel, J.L., 2014. Desorption kinetics of PAHs from aged industrial soils for availability assessment. *Sci Total Environ.* 470–471:639–645.

Cébron, A., Louvel, B., Faure, P., France-Lanord, C., Chen, Y., Murrell, J.C., Leyval, C., 2011. Root exudates modify bacterial diversity of phenanthrene degraders in PAH-polluted soil but not phenanthrene degradation rates. *Environ. Microbiol.* 13(3):722–736.

Connaughton, D.F., Stedinger, J.R., Lion, L.W., Shuler, M.L., 1993. Description of time-varying desorption kinetics: release of naphthalene from contaminated soils. *Environ. Sci. Technol.* 27(12) 2397–403.

Cornelissen, G., Van Noort, P.C.M., Govers, H.A.J., 1997. Desorption kinetics of chlorobenzenes, polycyclic aromatic hydrocarbons, and polychlorinated biphenyls: Sediment extraction with Tenax® and effects of contact time and solute hydrophobicity. *Environ Toxicol Chem.* 16(7):1351–1357.

Das, S., & Varma, A., 2011. Roles of enzymes in maintaining soil health. *In*: Shukla, G., Varma, A., eds, *Soil Enzymology,* 1st ed, Vol 22 – Soil Biology. Springer-Verlag, Berlin, Heidelberg, Germany, pp. 25-42.

Davin, M., Starren, A., Deleu, M., Lognay, G., Colinet, G., Fauconnier, M-L., 2018. Could saponins be used to enhance bioremediation of polycyclic aromatic hydrocarbons in aged-contaminated soils? *Chemosphere.* 194:414–421.

Ehlers, L.J., & Luthy, R.G., 2003. Contaminant bioavailability in improving risk assessment and remediation rests on better understanding bioavailability. *Environ Sci Technol.* 37:295–302.

Hall, J., Soole, K., Bentham, R., 2011. Hydrocarbon phytoremediation in the family *Fabaceae*-a review. *Int. J. Phytoremediation.* 13(4):317–332.

Hamdi, H., Benzarti, S., Aoyama, I., Jedidi, N., 2012. Rehabilitation of degraded soils containing aged PAHs based on phytoremediation with alfalfa (*Medicago sativa* L.). *Int. Biodeterior. Biodegrad.* 67:40-47.

Haritash, A.K., & Kaushik, C.P., 2009. Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review. *J. Hazard. Mater.* 169(1–3):1–15.

Hoagland, D.R., & Arnon, D.I., 1950. *The water-culture method for growing plants without soil.* California Agricultural Experiment Station, Circular-347.

Institut National de l’Environnement Industriel et des Risques (INERIS), 2005. Hydrocarbures Aromatiques Polycyliques - Guide méthodologique - Acquisition des données d’entrée des modèles analytiques ou numériques de transferts dans les sols et les eaux souterraines. 53(9):1689–1699. doi:10.1017/CBO9781107415324.004.

ISO 11465:1993 cor 1994 - Soil quality - Determination of dry matter and water content on a mass basis - Gravimetric method.

ISO 13877:1998 Soil quality - Determination of polynuclear aromatic hydrocarbons - Method using high -performance liquid chromatography.

ISO 11261:1995 Soil quality - Determination of total nitrogen - Modified Kjeldahl method.

ISO 14235:1998 Soil quality - Determination of organic carbon by sulfochromic oxidation.

Johnsen, A.R., Wick, L.Y., Harms, H., 2005. Principles of microbial PAH-degradation in soil. *Environ Pollut.* 133:71–84.

Kass, R.E., & Raftery, A.E., 1995. Bayes Factors. *J. Am. Stat. Assoc.* 90(430):773–795.

Kobayashi, T., Kaminaga, H., Navarro, R.R., Iimura, Y., 2012. Application of aqueous saponin on the remediation of polycyclic aromatic hydrocarbons-contaminated soil. *J. Environ. Sci. Health. - Part A Tox/Hazard Subst. Environ. Eng.* 47:1138–1145.

Kregiel, D., Berlowska, J., Witonska, I., Antolak, H., Proestos, C., Babic, M., Babic, L., Zhang, B., 2017. Saponin-Based, Biological-Active Surfactants from Plants. In: Najjar R., ed, *Application and Characterization of Surfactants,* IntechOpen. DOI: 10.5772/65591

Laha, S., Tansel, B., Ussawarujikulchai, A., 2009. Surfactant-soil interactions during surfactant-amended remediation of contaminated soils by hydrophobic organic compounds: A review. *J Environ. Manage.* 90:95–100.

Louvel, B., 2010. Etude en microcosmes de l’effet du ray-gras et de ses exsudats racinaire sur la dissipation des HAP et les communautés bactériennes dégradantes. Université de Lorraine, Cours Léopold, Nancy, France.

Martin, B.C., George, S.J., Price, C.A., Ryan, M.H., Tibbett, M., 2014. The role of root exuded low molecular weight organic anions in facilitating petroleum hydrocarbon degradation: Current knowledge and future directions. *Sci Total Environ.* 472: 642–653.

Oleszek, W., & Bialy, Z., 2006. Chromatographic determination of plant saponins-An update (2002-2005). *J. Chromatogr. A.* 1112:78–91.

Ouvrard, S., Chenot, E.D., Masfaraud, J.F., Schwartz, C., 2013. Long-term assessment of natural attenuation: Statistical approach on soils with aged PAH contamination. *Biodegradation*. 24(4):539-548.

Ouvrard, S., Leglize, P., & Morel, J.L., 2014. PAH Phytoremediation: Rhizodegradation or Rhizoattenuation? *Int. J. Phytoremediation* 16(1), 46–61.

Prague, M., Diakite, A., Commenges, D., 2012. Package ’marqLevAlg’ - Algorithme de Levenberg-Marquardt en R : Une alternative à ’optimx’ pour des problèmes de minimisation. 1ères Rencontres R, Bordeaux, France. <hal-00717566>

Richardson, S.D., & Aitken, M.D., 2011. Desorption and bioavailability of polycyclic aromatic hydrocarbons in contaminated soil subjected to long-term *in situ* biostimulation. *Environ Toxicol. Chem.* 30(12):2674–2681.

Semple, K.T., Morriss, A.W.J., Paton, G.I., 2003. Bioavailability of hydrophobic organic contaminants in soils: fundamental concepts and techniques for analysis. *Eur. J. Soil. Sci.* 54:809–818.

Semple, K.T., Doick, K.J., Jones, K.C., Burauel, P., Craven, A., Harms, H., 2004. Peer Reviewed: Defining Bioavailability and Bioaccessibility of Contaminated Soil and Sediment is Complicated. *Environ. Sci. Technol.* 38(12):228A-231A.

Shaw, L., & Burns, R., 2005. Soil microbial activity. *In*: Bloem J, Hopkins DW, Benedetti A, eds., *Microbiological methods for assessing soil quality.* CABI Publishing, Cambridge, MA, USA.

Uroz, S., Courty, P.E., & Oger, P., 2019. Plant symbionts are engineers of the plant-associates microbiome. *Trends Plant Sci.* 24(10) 905-916.

Vincken, J.P., Heng, L., de Groot, A., Gruppen, H., 2007. Saponins, classification and occurrence in the plant kingdom. *Phytochemistry*. 68:275–297.

Von Lau, E., Gan, S., Ng, H.K., Poh, P.E., 2014. Extraction agents for the removal of polycyclic aromatic hydrocarbons (PAHs) from soil in soil washing technologies. *Environ Pollut.* 184:640–649.

Wei, S., & Pan, S., 2010. Phytoremediation for soils contaminated by phenanthrene and pyrene with multiple plant species. *J. Soils Sediments* 10, 886-894.

Yu L, Duan L, Naidu R, & Semple KT. 2018. Abiotic factors controlling bioavailability and bioaccessibility of polycyclic aromatic hydrocarbons in soil: Putting together a bigger picture. *Sci. Total Environ*, 613–614, 1140–1153.

8. Supplementary material

Supplementary table 2. PAHs total initial concentrations and PAHs extracted amounts after different times of extraction by Tenax® beads. Values were used to calculate remaining sorbed fractions for each time of extraction, according to equation (1).

	time (h)	N	Ace	Fle	Phen	Anthr	F	Pyr	BaA
Total concentration (μg.g^{-1}DW)	0	28.9 ± 1.7	19.4 ± 1.2	12.5 ± 1.1	46.5 ± 5.5	16 ± 1.4	65.9 ± 7.1	45.6 ± 4.8	28.3 ± 3.6
Bioaccessible concentration (μg.g^{-1}DW)	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	1	0.22 ± 0.09	0.21 ± 0.05	0.27 ± 0.06	1.18 ± 0.27	0.91 ± 0.18	1.28 ± 0.75	1.18 ± 0.35	0.83 ± 0.26
	2	0.18 ± 0.08	0.16 ± 0.03	0.24 ± 0.04	1.04 ± 0.11	1.03 ± 0.20	1.46 ± 0.16	1.07 ± 0.12	0.85 ± 0.18
	4	0.25 ± 0.03	0.20 ± 0.01	0.30 ± 0.02	1.51 ± 0.22	1.21 ± 0.10	2.11 ± 0.29	1.53 ± 0.16	1.21 ± 0.10
	8	0.30 ± 0.09	0.24 ± 0.02	0.35 ± 0.06	1.53 ± 0.07	1.34 ± 0.05	2.17 ± 0.08	1.64 ± 0.07	1.58 ± 0.10
	16	0.30 ± 0.13	0.22 ± 0.06	0.32 ± 0.06	1.58 ± 0.27	1.47 ± 0.32	2.08 ± 0.42	1.62 ± 0.29	1.54 ± 0.32
	24	0.22 ± 0.07	0.19 ± 0.04	0.30 ± 0.07	1.56 ± 0.53	1.44 ± 0.35	1.90 ± 0.12	1.33 ± 0.08	1.36 ± 0.13
	48	0.42 ± 0.05	0.29 ± 0.04	0.41 ± 0.03	1.79 ± 0.11	1.70 ± 0.44	2.54 ± 0.29	1.88 ± 0.11	2.00 ± 0.21
	72	0.43 ± 0.08	0.34 ± 0.08	0.45 ± 0.02	2.04 ± 0.17	1.99 ± 0.27	2.97 ± 0.24	2.24 ± 0.24	2.35 ± 0.20
	96	0.34 ± 0.04	0.25 ± 0.07	0.37 ± 0.05	1.81 ± 0.20	1.51 ± 0.23	2.55 ± 0.31	1.77 ± 0.25	1.98 ± 0.24

(continued)

	time (h)	**Chrys**	**BbF**	**BkF**	**BaP**	**DBahA**	**BghiP**	**IcdP**
Total concentration (µg.g^{-1}DW)	0	32.4 ± 4.0	23.1 ± 3.3	11.8 ± 1.6	18.3 ± 2.6	2.9 ± 0.1	14.1 ± 3.6	15 ± 2.6
Bioaccessible concentration (µg.g^{-1}DW)	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	1	1.12 ± 0.38	0.50 ± 0.21	0.24 ± 0.08	0.33 ± 0.10	0.06 ± 0.03	0.22 ± 0.08	0.26 ± 0.10
	2	1.26 ± 0.40	0.44 ± 0.12	0.20 ± 0.06	0.23 ± 0.05	0.04 ± 0.00	0.12 ± 0.02	0.12 ± 0.03
	4	1.75 ± 0.12	0.73 ± 0.08	0.33 ± 0.03	0.40 ± 0.03	0.04 ± 0.01	0.16 ± 0.03	0.17 ± 0.03
	8	2.27 ± 0.15	1.13 ± 0.17	0.55 ± 0.06	0.66 ± 0.05	0.07 ± 0.01	0.25 ± 0.07	0.30 ± 0.09
	16	2.25 ± 0.56	1.28 ± 0.23	0.51 ± 0.13	0.62 ± 0.18	0.06 ± 0.02	0.23 ± 0.06	0.29 ± 0.09
	24	2.06 ± 0.41	0.99 ± 0.15	0.46 ± 0.05	0.57 ± 0.06	0.06 ± 0.01	0.24 ± 0.03	0.26 ± 0.02
	48	2.77 ± 0.34	1.52 ± 0.16	0.76 ± 0.03	0.95 ± 0.07	0.12 ± 0.03	0.43 ± 0.04	0.54 ± 0.06
	72	3.42 ± 0.24	1.73 ± 0.17	0.9 ± 0.07	1.14 ± 0.06	0.17 ± 0.06	0.51 ± 0.05	0.63 ± 0.04
	96	2.98 ± 0.27	1.42 ± 0.17	0.8 ± 0.11	0.95 ± 0.11	0.13 ± 0.02	0.32 ± 0.09	0.46 ± 0.06

Supplementary table 3. BIC values calculated for each desorption model of each PAH, according to equation (2).

	Model			
PAHs	**1 order**	**1 order - 2 compartment**	**1 order - 3 compartment**	**Site distribution**
N	-422*	-411	-401	-352
Ace	-428*	-411	-396	-337
Fle	-385*	-368	-372	-259
Phen	-362*	-313	-350	-237
Anthr	-249*	-242	-237	-141
F	-356*	-343	-351	-244
Pyr	-356*	-341	-346	-234
BaA	-313*	-302	-303	-202
Chrys	-285*	-276	-277	-179
BbF	-310*	-303	-301	-214
BkF	-318*	-312	-310	-232
BaP	-336*	-334	-317	-253
DBahA	-286	-277	-291*	-265
BghiP	-352	-349	-356*	-297
IcdP	-340	-331	-345*	-291

*smallest calculated BIC values, for each PAH.

Part 3

Temporal evolution of PAHs bioaccessibility in an aged-contaminated soil during the growth of two *Fabaceae*

1. Foreword

The data presented in this section were acquired during the testing of *Medicago sativa* L. and *Trifolium pratense* L. in more realistic conditions.

Several sets of data were collected through a one-year rhizoremediation trial. An aged-contaminated soil was cultured with each plant-type (*Medicago sativa* L. or *Trifolium pratense* L.) and compared to unplanted soil. Residual and bioaccessible PAHs contents were measured at the end of each incubation period (3, 6, and 12 months), respectively constituting two datasets. Because the experimental soil was different from the soil described in Part 1 and Part 2, a bioaccessibility measurement protocol also had to be adapted to this soil before acquiring the PAHs bioaccessible contents. The process of this adaptation, though similar to the one exposed in Part 2, will be exposed hereafter.

Finally, the plants biomass was measured to compare the performances of both tested species on the aged-contaminated soil.

All datasets (i.e. PAHs total residual contents, PAHs bioaccessible contents, and plants biomass) were discussed in a publication (Davin *et al.,* 2020). The content of this publication is exposed hereafter.

Reference

Davin, M., Renard, E., Lefébure, K., Colinet, G., Fauconnier, M.-L., 2020. Temporal evolution of PAHs bioaccessibility in an aged-contaminated soil during the growth of two *Fabaceae*. *Int. J. Environ. Res. Public Health*, 17(11), 4016. https://doi.org/10.3390/ijerph17114016

2. Abstract

Polycyclic aromatic hydrocarbons (PAHs) are health-concerning organic compounds that accumulate in the environment. Bioremediation and phytoremediation are studied to develop eco-friendly remediation techniques. In this study, the effects of two plants (*Medicago sativa* L. and *Trifolium pratense* L.) on the PAHs' bioaccessibility in an aged-contaminated soil throughout a long-term rhizoremediation trial was investigated. A bioaccessibility measurement protocol, using Tenax® beads, was adapted to the studied soil. The aged-contaminated soil was cultured with each plant type and compared to unplanted soil. The bioaccessible and residual PAH contents were quantified after 3, 6 and 12 months. The PAHs' desorption kinetics were established for 15 PAHs and described by a site distribution model. A common Tenax® extraction time (24 h) was established as a comparison basis for PAHs bioaccessibility. The rhizoremediation results show that *M. sativa* developed better than *T. pratense* on the contaminated soil. When plants were absent (control) or small (*T. pratense*), the global PAHs' residual contents dissipated from the rhizosphere to 8% and 10% of the total initial content, respectively. However, in the presence of *M. sativa*, dissipation after 12 months was only 50% of the total initial content. Finally, the PAHs' bioaccessible content increased more significantly in the absence of plants. This one-year trial brought no evidence that the presence of *M. sativa* or *T. pratense* on this tested aged-contaminated soil was beneficial in the PAHs remediation process, compared to unplanted soil.

3. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are persistent organic compounds of hydrophobic nature that are composed of fused rings in angular, linear or clustered arrangements (Ghosal *et al.*, 2016). PAHs mainly form during incomplete combustion, which is frequent in natural phenomena (volcanic eruption, forest fires) but also anthropogenic activities (car exhaustion, waste burning or domestic and industrial activities) (Dhar *et al.*, 2019). Because of their potential (geno)toxicity and heavy presence in former industrial areas (Keith, 2015), PAHs have been the centre of many remediation studies over the past decades.

On the one hand, many researchers have focused on PAH biodegradation pathways, which have been thoroughly reviewed (Ghosal *et al.*, 2016; Nzila, 2018; Dhar *et al.*, 2019). When it comes to the environmental influence on hydrophobic organic compounds' biodegradation, it is now well-known that the most important limiting factor is their bioaccessibility (Johnsen *et al.*, 2005), i.e., the availability of a chemical to "cross an organism's cellular membrane from the environment, if the organism has access to the chemical", as defined by Semple *et al.* (2004). The word "bioavailability" is extensively used in the literature but, by definition, the bioavailable fraction only refers to the chemical that is available "to cross an organism's cellular membrane from the environment at a given time", so the term "bioaccessibility" will be preferred in this paper. Indeed, for biodegradation to take place, the targeted pollutants must come into contact with the degrading microorganisms or their enzymes. This mostly takes place in the aqueous soil solution (Johnsen *et al.*, 2005). However, hydrophobic compounds, such as PAHs, are prone to ageing. Such phenomena are caused by environmental components (such as soil organic or mineral matter) that physically or chemically segregate compounds, thus lowering their presence in the aqueous solution and lowering their accessibility to the degrading agents. Ageing happens through two main mechanisms, sorption and diffusion, that have been extensively studied and reviewed (Semple *et al.*, 2003). The concept of bioaccessibility, compared to bioavailability, suggests that even a compound bound to a soil particle can become available to an organism if it is released into the organism's environment (Cui *et al.*, 2013). This is extremely important in the context of soil remediation because it means that treatments could influence the bioaccessible fraction of a pollutant.

On the other hand, two types of environmentally friendly remediation technologies are being developed, bioremediation and phytoremediation, which rely on the use of living microorganisms or plants to remediate pollutions. Even if they tend to be referred to as different technologies, they cannot be considered separately when applied in soil as close interactions exist between plants and microorganisms in all the soil's compartments (solid, liquid, and gas). The use of these interactions as a way to enhance the PAHs' biodegradation is named rhizoremediation and is based on the observation that the rhizosphere creates favorable chemical and physical conditions for the soil microbiota to thrive (Reichenauer & Germida, 2008). It has been hypothesized that this rhizospheric effect is a combination of physical and chemical positive effects, as roots are believed to: (i) facilitate contact between soil particles and the microbiota (Ouvrard *et al.,* 2014); (ii) increase soil oxygenation, which initiates aerobic metabolic pathways; (iii) exudate sugars, amino acids and organic acids, which serve as sources of energy for the microbiota (Alagić *et al.,* 2015); (iv) release secondary metabolites with structural analogy to PAHs, which could induce microbial catabolic genes and co-metabolism (Reichenauer & Germida, 2008); and (v) enhance pollutants' bioaccessibility. Indeed, some studies suggest that some compounds exuded by roots could desorb hydrocarbons from soil particles (Martin *et al.,* 2014). Besides, secondary metabolites are a collection of structurally different compounds (terpenes, nitrogen-containing products, phenolic compounds) among which some exhibit tensioactive properties (Taiz and Zeiger, 2002). For example, saponins are a diverse group of molecules composed of non-sugar aglycones coupled to sugar chain units, which gives them surface-active properties (Oleszek & Bialy, 2006). In a previous study (Davin *et al.,* 2019), we hypothesized that exudates from two *Fabaceae* (*Medicago sativa* L. or *Trifolium pratense* L.) could, as part of the rhizospheric effect, enhance the PAHs' bioaccessibility in an aged-contaminated soil, and thus enhance the PAHs' biodegradation. The results showed that a single-dose addition of root exudates to an aged-contaminated soil in a microcosm incubation experiment (4 weeks), did not enhance the PAHs' bioaccessibility nor dissipation. Given that plant root exudates are released at continuous rates into the environment (Canarini *et al.,* 2019), the following study was designed as a way to evaluate whether the prolonged presence of living *Medicago sativa* L. or *Trifolium pratense* L. on PAH aged-contaminated soil could enhance the PAHs' bioaccessibility and, hence, facilitate their dissipation.

The tested plants were chosen for the following reasons. (i) Due to their symbiotic relationship with nitrogen-fixating bacteria, *Fabaceae* members have a better potential to grow on disturbed soils that often present unfavorable conditions to plant growth. Therefore, the most common *Fabaceae* genera (such as *Medicago* sp or *Trifolium* sp) are encountered on various terrestrial environments, and very often in open and disturbed land (Hall *et al.,* 2011). (ii) Saponins are present in a large variety of plants, including members of the *Fabaceae* family (Sparg *et al.,* 2004). (iii) They have already been highlighted as good phytoremediation candidates (Hall *et al.,* 2011) through other phytoremediation studies; thus, this experiment could bring original insight to the mechanisms at work.

As the main objective of the study was to assess the PAHs' bioaccessibility in an aged-contaminated soil throughout a long-term rhizoremediation trial, the first step was to adapt a comparative bioaccessibility measuring protocol (using Tenax® beads) to the experimental soil. Therefore, PAH desorption kinetics were measured for the soil and modelled in order to assess a common extraction time for all PAHs. The second step was then to apply the protocol to measure the PAHs' bioaccessibility in an aged-contaminated soil that had been in the presence of *Medicago sativa* L. or *Trifolium pratense* L., for 3, 6 or 12 months, compared to unplanted soil. The residual PAH contents were also measured in soil at the end of each culture period.

4. Materials and Methods

4.1. Soil material

The aged-contaminated soil used for this study was sampled on a brownfield (Marchienne-au-Pont, Belgium). The coordinates are 50°24′51.4″ N 4°24′39.1″ E. The site hosted a steel company from 1863 to 2012 and has been exposed to PAHs and trace elements. Soil was sampled, sieved through an 8 mm sieve, allowed to dry in ambient air, and stored in sealed boxes until further use. Before the experiments, the contents of 15 PAHs were determined for a total of 917 ± 146 µg g^{-1} DW (Table 7). These PAHs are part of the 16 PAHs on the American Environmental Protection Agency (EPA) watch list. The sixteenth PAH compound (acenaphtylene) was not detected in the experimental soil. From now on, the term “total PAHs” will designate the 15 PAHs detailed in Table 7. PAHs were also grouped in categories: ∑2–3 rings or light molecular weight PAHs of three rings or less (N, Ace, Fle, Phen and Anthr), ∑4 rings or intermediate molecular weight PAHs of four rings (F and Pyr), ∑4–6 rings or heavy molecular weight PAHs of four rings or more (BaA; Chrys, BbF, BkF, BaP, DBahA, BghiP and IcdP), and ∑all or total PAHs (N to IcdP) (INERIS, 2005). The soil was also presented with metal contamination (541, 171, 1.39, 357, and 3373 µg g^{-1} DW of Cr, Cu, Hg, Pb, and Zn, respectively) but not petroleum hydrocarbons, PCBs, or BTEXs. The particle size distribution (75% sand, 19% silt, 6% clay) identified the soil as loamy sand, the pH_{H_2O} was 10.0, and the total organic carbon was 18.9 ± 0.22% (w/w). These last two parameters were very high compared to values encountered in uncontaminated soils.

4.2. PAHs bioaccessibility measurement

The PAHs’ bioaccessibility measurement protocol was developed based on a modelling technique previously described and used on a different aged-contaminated soil (Davin *et al.*, 2019) but it will be reminded hereafter. The objective was to determine the time of contact between the soil solution and the Tenax® beads (which serve as surrogate for the soil microbiota) that would extract the bioaccessible fraction of PAHs in the aged-contaminated soil.

Table 7. Experimental soil polycyclic aromatic hydrocarbons' (PAHs) initial contents.

PAHs	**Abbreviation**	**µg g^{-1} DW**
Naphthalene	N	20.2 ± 2.4
Acenaphthene	Ace	1.0 ± 0.4
Fluorene	Fle	5.1 ± 0.9
Phenanthrene	Phen	45.5 ± 7.2
Anthracene	Anthr	24.1 ± 3.6
Light PAHs	**∑2-3 rings**	**95.9 ± 12.2**
Fluoranthene	F	139 ± 36.6
Pyrene	Pyr	117 ± 20.5
Intermediate PAHs	**∑4 rings**	**256 ± 47.9**
Benzo[a]anthracene	BaA	79.2 ± 10.5
Chrysene	Chrys	73.6 ± 8.5
Benzo[b]fluoranthene	BbF	96.0 ± 19.4
Benzo[k]fluoranthene	BkF	48.1 ± 5.0
Benzo[a]pyrene	BaP	95.2 ± 15.6
Dibenzo[ah]anthracene	DBahA	12.1 ± 1.3
Benzo[ghi]perylene	BghiP	66.3 ± 25.3
Indeno[123-c,d]pyrene	IcdP	94.3 ± 21.7
Heavy PAHs	**∑4-6 rings**	**565 ± 90.0**
Total PAHs	**∑all**	**917 ± 146**

Values are mean ± confidence interval (α=5%, n=5)

PAHs desorption kinetics

In order to compare the PAHs' bioaccessibility throughout time and after different treatments, a comparison protocol was adapted from Cornelissen *et al.* (1997) and Barnier *et al.* (2013); then a specific extraction time, representative of the bioaccessible fraction, was determined for the studied soil. First, the desorption kinetics of all PAHs in the studied soil were measured five times: 2.0 g of soil were weighed into glass centrifuge tubes with 0.5 g of Tenax® beads (60–80 mesh) and 50 mL of an aqueous solution (0.01 M $CaCl_2$ and 0.003 M NaN_3 as biocides to prevent PAH degradation). The tubes were agitated for 1, 2, 4, 8, 16, 24, 48, 72 or 96 h on a rotary device (40 cycles min^{-1}) and centrifuged (10 min; 2000 × g) to separate the Tenax® beads from the soil. The floating beads were collected by vacuum filtration and sorbed PAHs were extracted from Tenax® beads by three repetitions of a 60 min sonication in presence of 20 mL of a 50:50 (v/v) n-hexane: acetone mixture. The combined organic phases were replaced with acetonitrile using a rotative evaporation device. The final acetonitrile extract was weighed for volume determination and analyzed for PAHs.

After this, the remaining PAH sorbed fractions in soil were calculated as follows:

$$\frac{S_t}{S_0} = \frac{C_{tot_{in}} - C_{ext_t}}{C_{tot_{in}}} \quad (4)$$

where $C_{tot\ in}$ is the total initial PAH concentration in the soil [µg g^{-1} DW]; $C_{ext\ t}$ is the amount of PAH extracted by Tenax® beads after t hours of contact [µg g^{-1} DW]; S_t is the sorbed fraction of compound remaining after t hours of extraction; and S_0 is the initial sorbed fraction, assumed to be the total initial PAH concentration.

PAHs desorption modelling

Several desorption models were tested to describe the PAHs desorption data (Table 8). Models were generated using R 3.4.3. and the following packages: “minpack.lm”, “AICcmodavg”, and “plotrix”. The Levenberg-Marquardt algorithm was used to minimize squared residuals between the experimental and calculated values (Prague *et al.,* 2012). The Bayesian information criterion (BIC) was also calculated to select the best model for each PAH as follows:

$$BIC = k.\ln(n) - 2.\,ln(L) \quad (5)$$

where k is the number of parameters of a model, n is the number of data points, and L the maximized value of a likelihood function. The R function is BIC (model_iner2).

Table 8. Desorption models tested to describe the measured desorption kinetics of PAHs in the experimental soil. Models were adjusted using the Levenberg–Marquardt algorithm (Prague *et al.*, 2012).

First-order model	**(1 parameter)**
$\frac{S_t}{S_0} = e^{-kt}$	
First-order two-compartment model	**(4 parameters)**
$\frac{S_t}{S_0} = F_{rap} \times e^{-k_{rap}t} + F_{slow} \times e^{-k_{slow}t}$ $F_{rap} + F_{slow} = 1$	
First-order three-compartment model	**(6 parameters)**
$\frac{S_t}{S_0} = F_{rap} \times e^{-k_{rap}t} + F_{int} \times e^{-k_{int}t} + F_{slow} \times e^{-k_{slow}t}$ $F_{rap} + F_{int} + F_{slow} = 1$	
Site distribution model	**(2 parameters)**
$\frac{S_t}{S_0} = \left(\frac{\beta}{\beta + t}\right)^{\alpha}$	

PAHs desorption parameters

The best models describing the PAH desorption kinetics were used to determine a common extraction time (t_{ex}) for bioaccessibility measurement, which is the time for the most accessible PAH fraction to equilibrate with Tenax® beads. In the models, it represents the time in which the slope closes down to zero. The slope limit was arbitrarily set to 10^{-3} and successive approximations were made according to the following equation:

$$\frac{y_{t_{ex}-24}-y_{t_{ex}}}{24} \leq 0.001 \qquad (6)$$

where y is the calculated value of a PAH desorption equation at different times and t_{ex} the extraction time [h].

The highest of all calculated t_{ex} was kept in the common comparative measuring protocol and used in the rhizoremediation experiment.

4.3. Rhizoremediation experiment

The rhizoremediation experiments were conducted in pots placed outdoors. Neither temperature nor sunshine time were controlled. Forty-five pots of dimension 10 × 10 × 15 cm each received 1 kg of dry experimental soil. Thirty pots were seeded with either 25 kg ha^{-1} (20 seeds per pot) of *Medicago sativa* L. (MS) or *Trifolium pratense* L. (TP) and 15 control samples (C) were left unplanted. Seeds were tested prior to the experiment and had a 100% germination rate. The pots were placed outdoors and arranged in a completely randomized block. The experiment lasted from April 2018 to April 2019, so that the plants would be exposed to a year of weather changes. During that year, the nearby weather station registered several drought episodes, a total of 169 dry days and 615 mm of cumulated precipitation instead of the normal 823 mm of this area (i.e., under average), meaning that, to prevent the plants' death, all 45 pots had to be regularly watered. Identical amounts of tap water were added to the (un)planted pots using a measuring cylinder. After 3, 6 and 12 months, respectively, 5 replicates of each modality were sacrificed for measurements. No sampling was performed in the winter because the plants would have slowed their activities. The PAHs' residual and bioaccessible contents were determined in the soil samples. In the planted soil samples, the analyses of the PAHs were performed on rhizospheric soil. This was achieved by carefully removing plants from the cultured soil, shaking all soil particles that were coming off easily and then collecting soil that was close to the plant roots by gently scraping it off. The presence/absence of plants and their length from roots to shoots were noted on planted samples. Plants were then carefully washed and dried. Their fresh biomass was determined through weighing, then plants were dried at 40 °C for 48 h and their dry biomass was determined through weighing. The soil samples will be referred to according to the type (MS, TP or C) and the length of time (3, 6 and 12 months) of the treatment they received.

4.4. Chemical analyses

Dry weight determination

The soil samples' dry weight determination was based on ISO 11465:1993 cor 1994.

Bioaccessible PAHs determination in soil samples

The bioaccessible PAH determination in the soil samples was realized on fresh (i.e., freshly sampled and undried) soil samples, as described in the PAHs desorption kinetics section. The time of contact between the soil and Tenax® beads through the aqueous solution was 24 h (see the PAHs desorption parameters paragraph of the results section for time choice).

Total PAHs determination in soil samples

The total PAH determination in the soil samples was based on ISO 13877:1998. The soils were chemically dried with an equivalent amount of anhydrous Na_2SO_4 and homogenized using a pestle and a mortar. The mixture was extracted for 16 h with dichloromethane on a Soxhlet device. The resulting organic phase was filtered on anhydrous Na_2SO_4, eliminated with a rotative evaporation device and replaced with n-hexane. Then, the extract was purified on basic Al_2O_3 before n-hexane was eliminated and replaced by acetonitrile. The final acetonitrile extract was weighed for volume determination and analyzed for PAHs.

PAHs analysis

The PAHs were analyzed in acetonitrile extracts of desorption kinetics, bioaccessible and residual samples according to ISO 13877:1998. Briefly 20 µL of PAHs in acetonitrile extract were injected on an Agilent reverse-phase C18 column (Eclipse PAH 4.6 × 250 mm, 5 µm) and eluted using acetonitrile and water, both acidified with formic acid (0.1% v/v). The elution flow rate was 1.5 mL min^{-1} and the acetonitrile/water gradient was: a linear increase from 50:50 to 75:25 from 0 to 15 min; a linear increase from 75:25 to 100:0 from 15 to 20 min; a 100:0 plateau from 20 to 40 min; and, finally, a linear decrease from 100:0 to 50:50 from 40 to 40.1 min, with a final isocratic hold of 2 min. The PAHs were detected fluorimetrically according to ISO 13877:1998 and their quantification was achieved using external standard calibration.

4.5. Statistics

All statistical analyses related to the rhizoremediation experiment were carried out using Minitab 18.0. The equality of variances were verified according to Levene's test, the data were analyzed by a general linear model or one-way analysis of variance, and mean values were compared by Tukey's test at the 5% confidence level.

5. Results

5.1. PAHs bioaccessibility measurement

Modelling PAHs desorption kinetics

After the soil samples were shaken for 1, 2, 4, 8, 16, 24, 48, 72 and 96 h in the presence of Tenax® beads, the PAH fractions that remained sorbed to the soil were calculated according to equation (4). Then, four desorption models (Table 8) were fitted on each PAH desorption dataset and on desorption data for each group of PAHs (∑2-3 rings, ∑4 rings, ∑4-6 rings and ∑all). Afterwards, the BIC values were calculated using R for each model of each dataset. As explained previously, the objective was to select one model that would best describe the datasets. Thus, the BIC values were used to choose the best-fitted model for each PAH and are available in Supplementary table 4. The site distribution model had the smallest BIC value for the most individual PAHs, except for Fle, Anthr and Chrys, and for each group of PAHs, except for the ∑2–3 rings group. In three cases the first-order three-compartment model obtained the smallest BIC values, and in one case it was the first-order two-compartment model that obtained the smallest BIC value. However, each time the BIC values were three or four units lower than the BIC values of the site distribution model. This means that the supplement of information brought by the first order three-compartment (or two-compartment) model is “positive but not strong” compared to the site distribution model (Kass & Raftery, 1995). Thus, the site distribution model was chosen to describe all individual and groups of PAHs’ desorption data (Figure 13) and to calculate the t_{ex} values. The parameters of the other models are not presented since they were not used afterwards.

PAHs desorption parameters

The site distribution models’ parameters (alpha and beta) are presented in Table 9 along with the t_{ex} values, calculated according to equation (6). The alpha values ranged from 4.40×10^{-4} to 4.41×10^{-3}, the beta values ranged from 2.17×10^{-7} h to 1.86 h, and the calculated extraction times were of 24 h for each compound and each group of PAHs. Therefore, a 24 h extraction time was used to determine the PAHs’ bioaccessible contents in the rhizoremediation experiment. As a comparison, when the PAH desorption kinetics were modelled on a different PAH aged-contaminated soil (Davin *et al.*, 2019) the common extraction time was 48 h.

5.2. PAHs rhizoremediation

Plant biomass

All plants’ seeds germinated well, which was expected given the 100% germination rate measured prior to the experiment and the fact that germination mobilizes a seed’s endosperm reserves (Müntz *et al.*, 2001). However at the end of each culture period, the presence or absence of plants in each pot was noted along with their length from roots to shoots. Throughout the experiment, and despite good germination, TP plants never developed well, especially compared to MS which developed dense root systems. The plants in one pot were dead in the TP_3, MS_6 and MS_12 samples and the plants of three pots were dead in the TP_12 samples at the end of their respective culture period. Statistical analyses on the plants' dry weights were performed after square root transformation. An analysis of variance showed significant interactions between time (3, 6, or 12 months) and treatment (C, MS, or TP). The results show that MS plants developed statistically more biomass than the TP plants as soon as after three months, and at the end of each culture period (Figure 14).

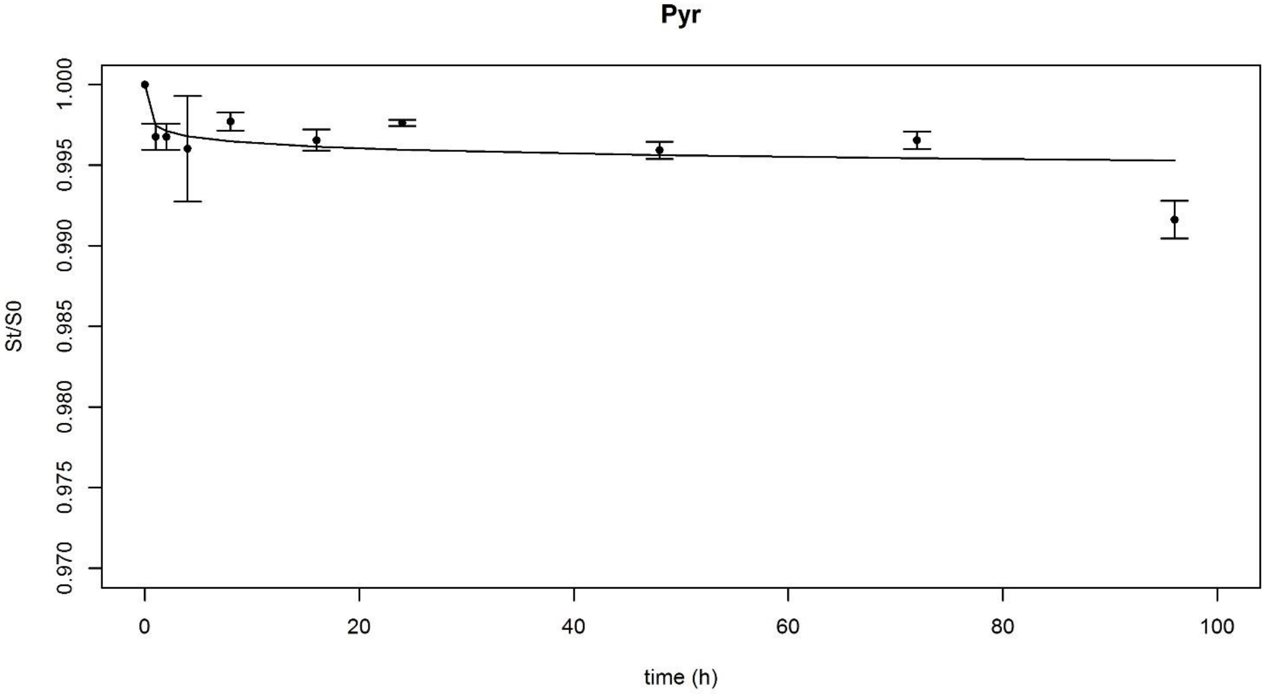


Figure 13. Example of desorption kinetic obtained using Tenax® beads (the data and the modelling are for pyrene). St/S0 is the remaining sorbed fraction according to extraction time. The dots are the data means ± confidence interval (α = 5%, n = 4 or 5); the line is the fitted site distribution model.

Table 9. Fitted parameters of the site distribution model for the different PAHs and t_{ex} values calculated according to equation (6).

	β (h)	**α (-)**	**t_{ex} (h)**
N	1.86×10^{0}	4.41×10^{-3}	24
Ace	7.30×10^{-2}	3.72×10^{-3}	24
Fle	1.06×10^{-1}	2.05×10^{-3}	24
Phen	4.39×10^{-2}	1.33×10^{-3}	24
Anthr	6.94×10^{-2}	1.53×10^{-3}	24
F	1.09×10^{-1}	1.37×10^{-3}	24
Pyr	4.92×10^{-3}	4.77×10^{-4}	24
BaA	8.84×10^{-2}	1.62×10^{-3}	24
Chrys	1.32×10^{-1}	2.20×10^{-3}	24
BbF	6.57×10^{-3}	1.04×10^{-3}	24
BkF	2.13×10^{-2}	1.41×10^{-3}	24
BaP	6.09×10^{-4}	6.84×10^{-4}	24
DBahA	2.17×10^{-7}	4.40×10^{-4}	24
BghiP	5.93×10^{-7}	5.38×10^{-4}	24
IcdP	6.73×10^{-5}	5.61×10^{-4}	24
∑2-3 rings	2.47×10^{-1}	1.95×10^{-3}	24
∑4 rings	4.97×10^{-2}	9.47×10^{-4}	24
∑4-6 rings	6.84×10^{-3}	1.07×10^{-3}	24
∑all	1.84×10^{-2}	1.11×10^{-3}	24

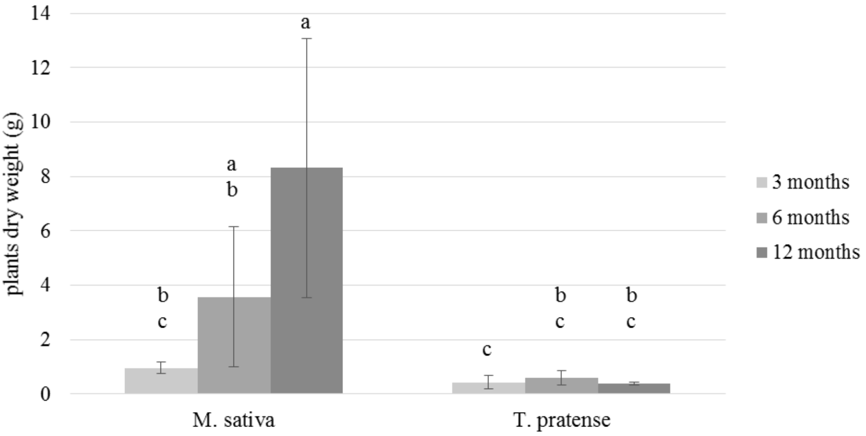


Figure 14. Plants dry weight (biomass) after each culture period. Within each group, bars that share the same letter are not significantly different ($p > 0.05$). The values are means ± confidence interval ($\alpha = 5\%$; $n = 5$; $n = 4$ for TP_3, MS_6 and MS_12; and $n = 2$ for TP_12).

PAHs bioaccessible and residual contents

Figure 15-Figure 18 show the (un)planted soil samples residual and bioaccessible PAH contents at different stages of the rhizoremediation experiment. The presented data focusses on the different groups of PAHs (∑2-3 rings in Figure 15, ∑4 rings in Figure 16, ∑4-6 rings in Figure 17 and ∑all in Figure 18) as they summarize and emphasize the observations made on individual PAHs data. Statistical analyses on the residual contents were performed after log10 transformation. An analysis of variance showed significant interactions between time (3, 6, or 12 months) and treatment (C, MS, or TP) on both the residual and bioaccessible contents.

In all figures, the first obvious observation is that all groups of PAHs' residual contents (Figure 15a, Figure 16a, Figure 17a, and Figure 18a) exhibited similar patterns within each type of treatment. The residual PAH contents in C samples significantly diminished throughout the whole experiment and the samples reached about 8% of the total initial content (∑all) after 12 months. On the other hand, the residual contents in the TP samples diminished rather abruptly after 3 months of culture to about 10% of the total initial content, then remain statistically similar after 6 and 12 months. The most surprising pattern was exhibited by the MS samples' residual contents. During the first 6 months, all the PAHs' residual contents was lowered to about 10% of their initial content. After 12 months, the ∑2–3 rings content was statistically higher than after 6 months, and the other groups of PAHs' residual contents clearly were not as low as after 6 months. The residual contents in the MS samples after 12 months were about 50% of the total initial content.

When it comes to bioaccessible PAHs contents (Figure 15b, Figure 16b, Figure 17b, and Figure 18b), different observations can be made, and, this time, the patterns were different between the PAH groups. First, the ∑4–6 rings and ∑all contents did not significantly differ with treatment nor time, suggesting that, whilst the residual content globally lowers in all samples, bioaccessibility remains similar. When it comes to the ∑2–3 rings and ∑4 rings bioaccessible contents, the statistical analysis shows that they increased with time but in a more significant way in C samples.

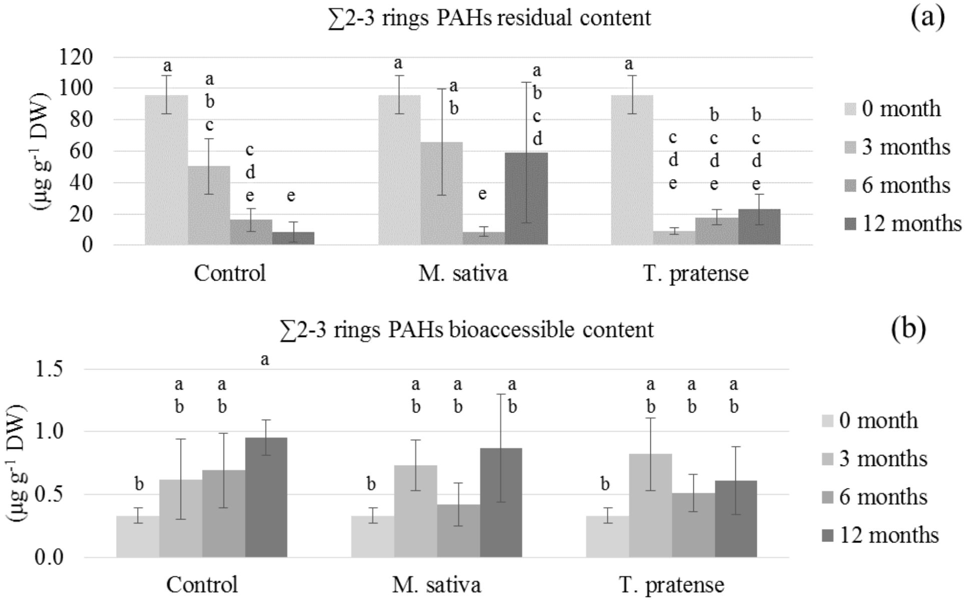


Figure 15. The light PAHs' (∑2–3 rings) (a) residual and (b) bioaccessible content of soils planted with *M. sativa* L. or *T. pratense* L. compared to unplanted control samples after different time periods. The values are means ± confidence interval (α = 5%, n = 5). There is a significant interaction between the type and the time of culture, so within each PAH fraction (residual or bioaccessible) sticks that share the same letter are not significantly different ($p > 0.05$).

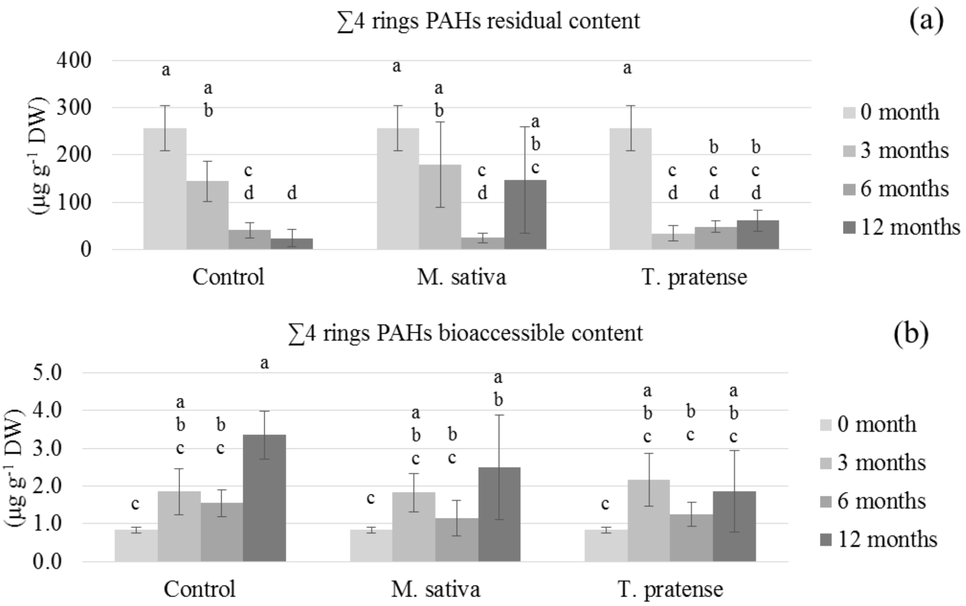


Figure 16. The intermediate PAHs (∑4 rings) residual (a) and bioaccessible (b) contents of soils planted with *M. sativa* L. or *T. pratense* L. compared to unplanted control samples after different time periods. Values are means ± confidence interval (α=5%, n=5). There is a significant interaction between the type and the time of culture so within each PAH fraction (residual or bioaccessible), sticks that share the same letter are not significantly different ($p > 0.05$).

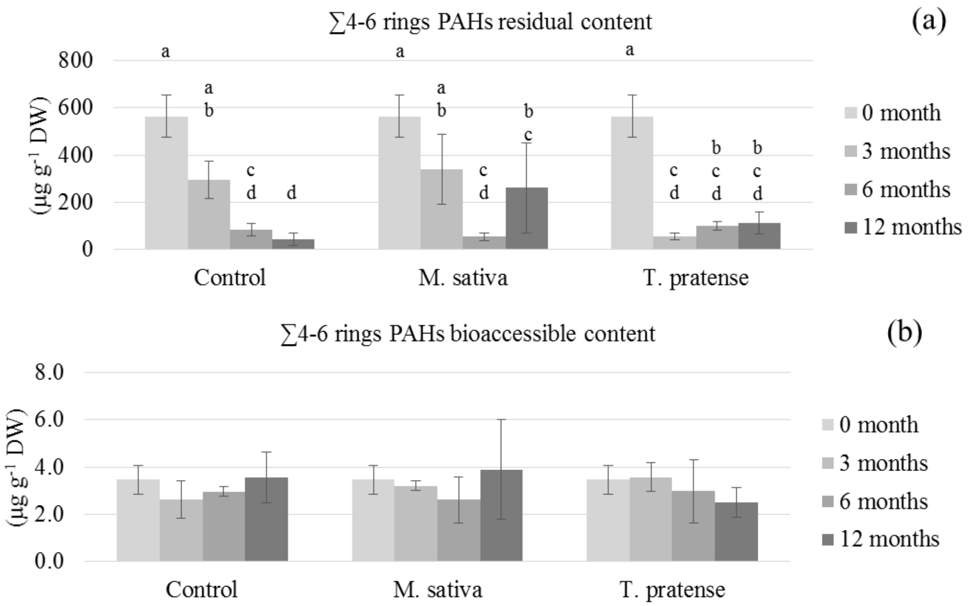


Figure 17. The heavy PAHs (∑4-6 rings) residual (a) and bioaccessible (b) contents of soils planted with *M. sativa* L. or *T. pratense* L. compared to unplanted control samples after different time periods. Values are means ± confidence interval (α=5%, n=5). There is a significant interaction between the type and the time of culture so within each PAH fraction (residual or bioaccessible), sticks that share the same letter are not significantly different ($p > 0.05$).

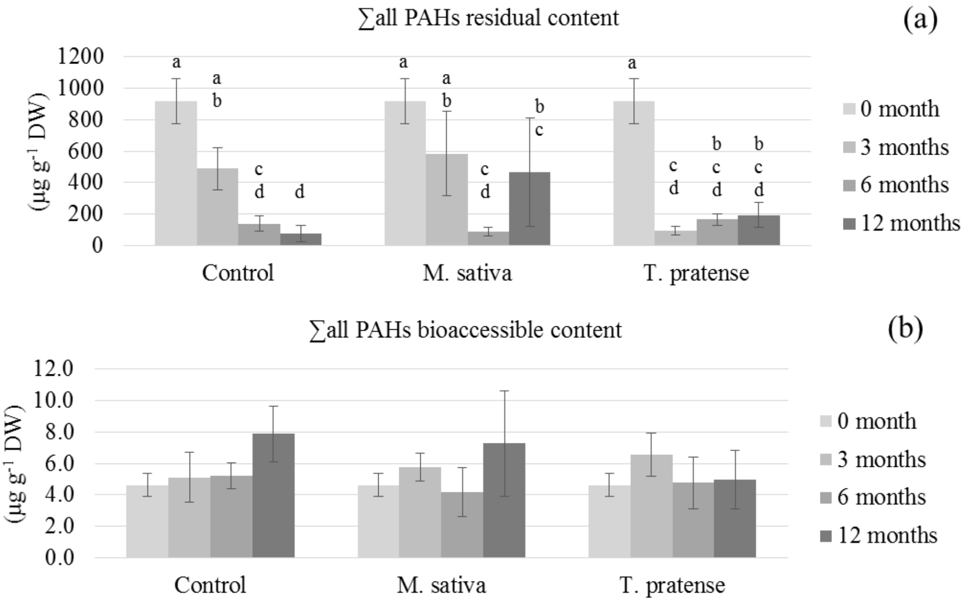


Figure 18. The total PAHs (∑all) residual (a) and bioaccessible (b) contents of soils planted with *M. sativa* L. or *T. pratense* L. compared to unplanted control samples after different time periods. Values are means ± confidence interval (α=5%, n=5). There is a significant interaction between the type and the time of culture so within each PAH fraction (residual or bioaccessible), sticks that share the same letter are not significantly different ($p > 0.05$).

6. Discussion

The objectives of this study were: (i) to adapt a comparative bioaccessibility measurement protocol using Tenax® beads to an aged-contaminated soil, and (ii) to follow the PAHs' bioaccessibility and residual contents in this soil in the presence of *Medicago sativa* L. or *Trifolium pratense* L., compared to unplanted control soil. The underlying hypothesis was that the continuous input of plant root exudates *in situ* could, as part of the rhizospheric effect, enhance the PAHs' bioaccessibility, and thereby render them more susceptible to biodegradation by soil microorganisms.

Desorption kinetics were measured and modelled for 15 PAHs individually and grouped in categories (light, intermediate, heavy, and total). Out of the four models, the site distribution model was chosen to calculate the minimal common Tenax® beads' extraction time (24 h). During the course of the experiment (April 2018 to April 2019), an ISO norm to "determine the potential and environmental availability of a contaminant" was published (ISO/TS 16751:2018). The Tenax® beads extraction protocol is overall similar to the one developed in this study and recommends a 20 h time of extraction, which is slightly less than calculated in this case. So even if the study was not conducted following a norm that came out while the study was ongoing, the protocols are very similar. Besides, the main objective of this protocol was to compare the bioaccessibility contents of soil that was submitted to different treatments, which was achieved.

Regarding the rhizoremediation experiment, it was conducted in the expectation of obtaining better PAH dissipation results. First of all, the *T. pratense* plants did not grow or last well in the experimental soil (Figure 14), even though *T. pratense* seeds germinated well, as previously mentioned. Secondly, and even if some of the *M. sativa* plants died during the experiment, they developed more biomass than *T. pratense*. These outcomes were compared to a few results previously reported in the literature and summarized in Table 10. The presented results show similarities in the way that PAHs do not seem to affect germination but can affect growth by either decreasing it or increasing it. Importantly, Smith *et al.* (2003) reported that *T. pratense* growth reduction could not have been foreseen by a traditional germination test. Therefore, the elevated amount of PAHs, although weathered, present in our experimental soil is likely to be responsible for the *T. pratense* plants decay on the long term.

The PAH levels were probably not the only factor that influenced the tested plants' growth. Indeed, brownfield soils that are in need for remediation rarely present with a single type of contamination, and it has been pointed out that the experimental soil had Cr, Cu, Hg, Pb, and Zn contaminations. Besides, the cultures were conducted on a very high pH (10.0). To the best of our knowledge, it is difficult to know whether *M. sativa* or *T. pratense* are tolerant to such elevated pH since this value is out of the usual working range encountered in traditional soil use, such as agriculture, and there is no information on that matter in the literature. The choice not to use amendments in this experiment originates from economic considerations. Indeed, many brownfields already lack management and remediation because of financial considerations. Some brownfields are considered worth the remediation investment, some are not. So, the experimental setup aimed at exploring and developing remediation techniques that are as low-cost and low-maintenance as possible, hence the initial choice to not use amendments. However, such growth conditions might have caused some of the plants to decay since an elevated pH lowers essential nutrient availability in soil solution (Genot *et al.*, 2009). Therefore, it would be interesting to repeat this rhizoremediation experiment by amending the soil with plant essential nutrients to enhance their growth (especially *T. pratense* L. in this case). But it is important to emphasize that the use of such soil in the experiment provided observations as to *M. sativa*'s and *T. pratense*'s capacity to enhance rhizoremediation in realistic and unoptimized conditions, and shows that there is still some research that needs to be conducted on the phytoremedation of soils presenting multiple types of contaminations. Finally, a possible explanation as to why *M. sativa* plants were less affected than *T. pratense* probably lies in the structural differences between the two tested plant species. Indeed *M. sativa* has a deep taproot which is a great adaptation to sandy soils, whereas *T. pratense* has a shallow and highly branched root system, which is not as efficient on a more sandy soil such as the experimental soil (which, as a reminder, identified as loamy sand).

Table 10. Comparison of germination and growing conditions and outcomes between a few published references and the presented experimental soil, for *Medicago sativa* L. and *Trifolium pratense* L.

Reference	**Tested plant(s)**	**Germination / growing conditions**	**Germination / growing outcomes**	**Germination / growing conditions in presented experimental soil (Table 7)**
Sverdrup et al. (2003)	*T. pratense* L.	Soil freshly spiked with Fle, Phen, F, and Pyr at individual concentrations up to 1000 mg kg^{-1} DW	No seed emergence inhibition; 20% plant growth inhibition starting at concentrations: 55 mg kg^{-1} DW (Fle), 37 mg kg^{-1} DW (Phen), 140 mg kg^{-1} DW (F), and 49 mg kg^{-1} DW (Pyr).	Individual concentrations of: 20.2 mg kg^{-1} DW (N), 5.1 mg kg^{-1} DW (Fle), 45.5 mg kg^{-1} DW (Phen), 139 mg kg^{-1} DW (F), 117 mg kg^{-1} DW (Pyr), 79.2 mg kg^{-1} DW (BaA), 73.6 mg kg^{-1} DW (Chrys)
Smith et al. (2006)	*T. pratense* L.	Soil spiked with seven PAHs and aged for four weeks (total concentration was 450 mg kg^{-1} DW after the ageing process)	Germination was not affected; Growth was significantly reduced (70%).	
		Aged-contaminated soil (total concentration of 16 PAHs was 5300 mg kg^{-1} DW)	Germination was not affected; Growth was significantly reduced (65%).	

(continued)

Reference	Tested plant(s)	Germination / growing conditions	Germination / growing outcomes	Germination / growing conditions in presented experimental soil (Table 7)
Henner et al. (1999)	*M. sativa* L.; *T. pratense* L.	Pure saturated solutions of N, Phen, F, Chrys and BaA	Similar germination levels as in the absence of PAHs.	Total concentration of 15 PAHs was 917 mg kg^{-1} DW.
		Aged-contaminated soil (total concentration of 16 PAHs was 1500 mg kg^{-1} DW)	Germination slowed (3-4 days) but reached similar levels as in uncontaminated soil; Plant growth was inhibited (80%) for *M. sativa*; No information for *T. pratense*.	
Afegbua and Batty (2018)	*M. sativa* L.	Soil spiked with Phen (300 mg kg^{-1} DW), F (200 mg kg^{-1} DW), and BaA (5 mg kg^{-1} DW) then aged for four weeks.	Shoots and roots dry biomass respectively increased by 110 and 40% when PAHs were mixed.	

Regarding the PAH residual contents from the rhizoremediation experiment, contrasting results have already been published in the literature and are summarized in Table 11. We should, however, mention that there were many more phytoremediation assays involving *M. sativa* L. than *T. pratense* L. Besides, many studies involving PAH phytoremediation were either performed on soil freshly spiked with PAHs (which often are only a few representative compounds such as BaA, Pyr or Phen), or on spiked soil that was allowed to age for a few weeks, and sometimes a few months. Fewer studies were performed on aged-contaminated soil such as the one used in this study, and, if such soil was experimented with, the growing conditions were controlled as trials often took place in greenhouses, or the initial PAH concentrations were sometimes much lower than for our tested experimental soil. Also, some of them lacked unplanted control to compare the PAH dissipation results. The experiment by Olson *et al.* (2007) is the most similar to the one in this study in terms of the PAHs' diversity, initial content, and final dissipation rates compared to an unplanted control. The authors hypothesized that the symbiosis relationship of the *Fabaceae* plants with rhizobia offered long-term advantages to the plants and their rhizosphere microbial community but have not observed a correlated raise in the PAH-degrading microbial community to corroborate their hypothesis.

However, and concerning the PAHs' residual contents presented in this study, several hypotheses were formulated to explain the unexpected fact that MS_12 residual contents were higher than the MS_6 contents. (i) The easiest would be to acknowledge the large natural variability of biological experiments. As a reminder, samples were sacrificed at the end of each culture period so data from increasing time periods do not represent the continuity of the same planted pots, meaning either the MS_6 or the MS_12 samples data could constitute an exception. But, since MS plants were statistically as developed after 12 months as after 6 months (Figure 14), similar (or lower) PAHs' residual contents were expected to be measured at the end of the experiment. (ii) PAHs could have been temporarily sequestered by plants and then released through roots decay. PAHs can be adsorbed onto the root cell membranes, as was reported for naphthalene with *M. sativa* roots by Schwab *et al.* (1998) and for phenanthrene and pyrene with *Lolium multiflorum* Lam. by Kang *et al.* (2010), who both concluded that the adsorbed amounts were linked to cell lipid contents. Besides, the fine roots of perennial plants continuously grow and die over time (Leigh *et al.*, 2002), with periods of either net production or net loss throughout the year, suggesting PAHs could have been released back to the soil because of root decay taking place during the second part of the experiment, which corresponds to the end of autumn and winter. (iii) Given the dry culture conditions (several droughts combined to a sandy draining soil) that plants endured, and the high capacity of *M. sativa* L. to draw water with dense and deep root systems, PAHs might actually have dissipated from the plants rhizosphere in MS samples (either by volatilization, degradation, or lixiviation) during the first 6 months, and, as plants roots grew denser, they might have vertically reached and retained more PAHs in their rhizosphere.

To summarize the PAH residual contents results, it can be stated that: (i) in a short time (3 months) the presence of *T. pratense* L. plants led to greater PAHs' dissipation than in the control and *M. sativa* L. samples, which tend to confirm *T. pratense* L.'s potential for phytoremediation, whilst *M. sativa* L. did not enhance PAHs dissipation compared to control samples. (ii) Dissipation in *T. pratense* L. samples was similar after 3, 6 and 12 months, regardless of the fact that many *T. pratense* L. samples plants died during the experimental period. (iii) After 6 months, dissipation in control samples was statistically similar to dissipation in planted samples, which was confirmed in the long-term (12 months) for *T. pratense* L. samples but not for *M. sativa* L. samples, which presented higher residual contents. If PAHs were dissipated through biodegradation mechanisms, it would mean that plants did not enhance biodegradation in the long term. However, if dissipation simply results from leaching and/or lixiviation, the slower dissipation in presence of *M. sativa* L. could be caused by roots preventing vertical migration by physically retaining soil particles or "pumping up" contaminated soil solution, which would be confirmed by the MS_12 residual contents. All the mentioned hypotheses could be investigated by repeating this experiment for another year, comparing data, and analyzing plants' PAH contents after shorter culture periods, (i.e., every month for a year) to follow more accurately the fate of PAHs in the presence of these plants.

Table 11. Comparison of phytoremediation conditions and outcomes between a few published references for *Medicago sativa* L. and *Trifolium pratense* L.

Reference	**Tested plant(s)**	**Phytoremediation conditions**	**Phytoremediation outcomes**
Fan et al. (2008)	*M. sativa* L.	Soil freshly spiked with Pyr (500 mg kg^{-1} DW).	6% better removal in the rhizosphere compared to the non-rhizosphere soil.
Hamdi et al. (2012)	*M. sativa* L.	Soil spiked with BaA (100 mg kg^{-1} DW) + 15-month landfarming (bioremediation process) had brought content down to 9 mg kg^{-1} DW. Then soil was planted 5 months in controlled conditions.	BaA content lowered to 4.3 mg kg^{-1} DW. No unplanted control to compare results.
Teng et al. (2011)	*M. sativa* L.	Agricultural weathered soil (total concentration of 16 PAHs was 10 mg kg^{-1} DW) was planted for 3 months.	45% lowering of the 16 PAHs mixture.
Olson et al. (2007)	*M. sativa* L.; *T. pratense* L.	Weathered soil (total concentration of 17 PAHs was 753 mg kg^{-1} DW) was planted 14 months in controlled conditions.	Total PAHs dissipation was not different from unplanted control samples, after 7 and 14 months.

The PAHs' bioaccessible results were compared to previously published information summarized in Table 12. References were chosen that presented various tested remediation techniques, similar bioaccessibility measurement protocols, and, of course, were performed on aged-contaminated soils. The reported remediation techniques in Table 12 are either phytoremediation, biostimulation (which enhances existing microorganisms' activity through the use of amendments or optimized conditions) that were applied through biopiles or composting, bioaugmentation (which inoculates specialized degrading strains to a soil), and chemical oxidation. The results vary in terms of residual PAHs' diminution, but these concentrations always decrease or remain similar. Also, lighter PAHs' contents (such as Phen) tend to decrease more than heavier PAHs' contents (such as BaA), which was not observed in the present experimental results. The bioaccessible PAHs' contents, however, show contrasting patterns. Posada-Baquero *et al.* (2019; 2020) reported that, generally speaking, techniques such as phytoremediation or biostimulation seem to lead to decreases in PAHs bioaccessible contents, whilst techniques that were more focused on influencing bioaccessibility, such as the addition of surfactants or bioaugmentation, seem to lead to increases in the PAHs' bioaccessible contents. However, the results reported by Medina *et al.* (2020) also showed an increase in PAHs bioaccessible contents after biostimulation was employed. A similar pattern was observed after chemical oxidation. The results presented in this paper are also in contradiction with the theory exposed by Posada-Baquero *et al.* (2019), even though the reported phytoremediation results are based on different plants. In the present paper, the PAHs' bioaccessible contents throughout the rhizoremediation trial show almost similar patterns for (un)planted soil samples (Figure 15b, Figure 16b, Figure 17b, and Figure 18b). The light and intermediate PAHs' bioaccessibility raised throughout the experiment but globally (∑all) remained unchanged. This suggests that there is no global effect of *M. sativa* L. nor *T. pratense* L. culture on bioaccessibility, which would mean that equilibrium balances unrelated to the plants presence or absence are filling the vacancy left by the dissipation of PAHs throughout the experiment. Therefore, it would seem reasonable to conclude that the tested *Fabaceae* do not enhance the PAHs' bioaccessibility compared to unplanted soil. However, the less significant rise of the light and intermediate PAHs' bioaccessibility in planted samples compared to control samples suggests that the *M. sativa* L. or *T. pratense* L. plants' presence actually slows the increase of bioaccessibility. According to Ouvrard *et al.* (2014), this would make sense considering that PAHs are hydrophobic compounds that tend to sorb on organic soil content, and part of the PAHs released in soil aqueous solution could have been sorbed onto plants exudates, explaining a less important increase of bioaccessibility in planted samples. The lowering of bioaccessibility might also be caused by interactions between the targeted pollutants and some surface-active compounds released from the plant roots into the rhizosphere. It has indeed been demonstrated that surface-active compounds (such as saponins) could form micelles that can enhance the PAHs apparent solubility in the environment (Zhou *et al.*, 2011). However, it has also been demonstrated that hydrophobic interactions can take place between soil particles and the surfactants (Laha *et al.*, 2009), meaning PAHs could be partitioned into micelles or hemimicelles bound to hydrophobic constituents of the rhizosphere, such as soil particles or even lipid membranes from the roots. A similar hypothesis has already been advanced in a previous study that aimed to increase PAHs' apparent solubility by washing an aged-contaminated soil with aqueous solutions of saponins from *Quillaja saponaria* Molina bark (Davin *et al.*, 2018). The results showed a less efficient extraction of PAHs if the surfactant concentration was too elevated. Such seclusion of PAHs away from biodegradation agents would thus explain why *M. sativa* L. and *T. pratense* L. presence in soil lowered the pollutants bioaccessibility instead of increasing it.

Table 12. Comparison of remediation conditions and outcomes between a few published references for PAHs residual and bioaccessible contents.

Reference	Initial soil concentrations	Remediation conditions	PAHs residual concentrations evolution	PAHs bioaccessible concentrations evolution
Posada-Baquero *et al.* (2019)	Phen and BaA concentrations were 843.10 and 56.5 mg kg^{-1} ; Phen and BaA bioaccessible concentrations were 0.75 and 0.10 mg kg^{-1}	5 months biostimulation in a biopile amended with urea and KH_2PO_4; No reported control	Phen diminished by over 94%; BaA diminished by about 35%	Phen diminished by almost 90 %; BaA diminished by 30%
	Phen and BaA concentrations were 197.10 and 4.12 mg kg^{-1} ; Phen and BaA bioaccessible concentrations were 0.42 and 0.20 mg kg^{-1}	60 days sunflowers phytoremediation in a greenhouse; No reported control	Phen diminished by over 97%; BaA diminished by about 46%	Phen diminished by over 86%; BaA diminished by 70%
	Phen and BaA concentrations were 36.7 and 0.64 mg kg^{-1} ; Phen and BaA bioaccessible concentrations were 0.23 and 0.03 mg kg^{-1}	60 days bioaugmentation with specialised strains; No reported control	Phen diminished by over 30%; BaA diminished by over 10%	Phen raised by over 140%; BaA raised by 300%
	Phen and BaA concentrations were 46.3 and 1.40 mg kg^{-1} ; Phen and BaA bioaccessible concentrations were 0.27 and 0.024 mg kg^{-1}	60 days bioaugmentation with specialised strains; No reported control	Phen diminished by 60%; BaA did not diminish	Phen raised by over 35%; BaA raised by over 200%

(continued)

Reference	**Initial soil concentrations**	**Remediation conditions**	**PAHs residual concentrations evolution**	**PAHs bioaccessible concentrations evolution**
Medina *et al.* (2020)	Aged-contaminated soil (PAHs concentration was 214 mg kg^{-1} and bioaccessible PAHs fraction was 1%)	Chemical oxidation with ammonium persulfate; No reported control	PAHs diminished by almost 30%	PAHs raised to a 19% fraction of remaining total PAHs
	Aged-contaminated soil (PAHs concentration was 151 mg kg^{-1} and bioaccessible PAHs fraction was 19%)	12 months incubation (served as control)	PAHs diminished by 25%	PAHs raised to a 30% fraction of remaining total PAHs
		12 months biostimulation through composting with amended goat manure	PAHs diminished by 33%	PAHs raised to a 56% fraction of remaining total PAHs
Posada-Baquero *et al.* (2020)	Aged-contaminated soil (PAHs concentration was 513 mg kg^{-1} and bioaccessible PAHs fraction were 60 and 40% for light and heavy PAHs, respectively)	210 days of sunflower phytoremediation in a greenhouse combined to a biosurfactant amendment after 75 days	Light and heavy PAHs respectively diminished by over 90 and 70% in (un)planted soil samples; Biosurfactant addition had no effect.	Light and heavy PAHs respectively diminished under 10 and around 10% in (un)planted soil samples; Biosurfactant addition enhanced all PAHs bioaccessible fractions in planted samples for a few days; At the end, bioaccessible fractions were similar in all samples

7. Conclusions

As a conclusion, the general rhizoremediation results suggest that when plants are small or absent, the PAHs' residual contents seem to globally dissipate faster from the rhizosphere and the bioaccessibility contents to increase a little faster (at least for light and intermediate PAHs). From a remediation point-of-view, it means this one-year trial brought no evidence that the presence of *M. sativa* L. or *T. pratense* L. on this aged-contaminated soil was beneficial on the PAHs' remediation process, compared to unplanted soil. However, from an environmental risk point-of-view, the slower dissipation but also bioaccessibility enhancement of PAHs in the presence of those plants could be used as a tool to prevent the migration of the contaminants towards more sensitive environmental compartments such as ground or even surface water.

Let us also point out here that contrasting observations have been made previously in the literature. First, as to the PAHs effects on *M. sativa* L. and *T. pratense* L. growth, whether cultures took place on freshly spiked or aged-contaminated soil, but also as to the effect of those plant types on PAHs remediation, this study added information to previously acquired data. Because it led to mitigated conclusions, it highlights the complexity of plant–soil–pollutant interactions and the fact that there might be antagonist events taking place within this system. It also points out the need to perform more phytoremediation experiments on a broad range of aged-contaminated soil types presenting different pedologic characteristics and different levels and types of contamination to try and predict the conditions in which plants might grow and enhance PAHs' remediation. Also, it points out the importance of a thoughtful selection of the plants to try and remediate the contaminated soils, as they are likely to be confronted with difficult growth conditions such as extreme pH, poor nutrient availability or inadequate soil drainage. Finally, we would like to insist that the parallel evaluation of both the PAHs' bioaccessible and residual contents, as was performed in this study, could bring new insights to the complexity of soil remediation trials in general, if they were to be realized more systematically.

8. References

Afegbua, S.L., & Batty, L.C., 2018. Effect of single and mixed polycyclic aromatic hydrocarbon contamination on plant biomass yield and PAH dissipation during phytoremediation. *Environ Sci Pollut Res.*, 25:18596–18603. https://doi.org/10.1007/s11356-018-1987-1

Alagić, S., Maluckov, B.S., & Radojičić, V.B., 2015. How can plants manage polycyclic aromatic hydrocarbons? May these effects represent a useful tool for an effective soil remediation? A review. *Clean Technol Environ.*, 17:597–614. https://doi.org/10.1007/s10098-014-0840-6

Barnier, C., Ouvrard, S., Robin, C., Morel, J.L., 2014. Desorption kinetics of PAHs from aged industrial soils for availability assessment. *Sci Total Environ.,* 470-471:639-645. https://doi.org/10.1016/j.scitotenv.2013.10.032

Canarini, A., Kaiser, C., Merchant, A., Richter, A., Wanek, W., 2019. Root exudation of primary metabolites: Mechanisms and their roles in plant responses to environmental stimuli. *Front Plant Sci.*, 10. https://doi.org/10.3389/fpls.2019.00157

Cornelissen, G., Van Noort, P.C.M., & Govers, H.A.J., 1997. Desorption kinetics of chlorobenzenes, polycyclic aromatic hydrocarbons, and polychlorinated biphenyls: Sediment extraction with Tenax® and effects of contact time and solute hydrophobicity. *Environ Toxicol Chem.,* 16(7):1351-1357. https://doi.org/10.1897/1551-5028(1997)016<1351:dkocpa>2.3.co;2

Cui, X., Mayer, P., Gan, J., 2013. Methods to assess bioavailability of hydrophobic organic contaminants: Principles, operations, and limitations. *Environ Pollut.,* 172:223–234. https://doi.org/10.1016/j.envpol.2012.09.013

Davin, M., Starren, A., Deleu, M., Lognay, G., Colinet, G., Fauconnier, M-L., 2018. Could saponins be used to enhance bioremediation of polycyclic aromatic hydrocarbons in aged-contaminated soils? *Chemosphere*, 194:414-421. https://doi.org/10.1016/j.chemosphere.2017.11.174

Davin, M., Starren, A., Marit, E., Lefébure, K., Fauconnier, M-L., Colinet, G., 2019. Investigating the Effect of *Medicago sativa* L. and *Trifolium pratense* L. Root Exudates on PAHs Bioremediation in an Aged-Contaminated Soil. *Water Air Soil Pollut.,* 230-296. https://doi.org/10.1007/s11270-019-4341-4

Dhar, K., Subashchandrabose, S.R., Venkateswarlu, K., Krishnan, K., Megharaj, M., 2019. Anaerobic Microbial Degradation of Polycyclic Aromatic Hydrocarbons: A Comprehensive Review. *In*: de Voogt P (ed.) *Reviews of Environmental Contamination and Toxicology*. Springer Nature, Switzerland. https://doi.org/10.1007/398_2019_29

Fan, S., Li, P., Gong, Z., Ren, W., He, N., 2008. Promotion of pyrene degradation in rhizosphere of alfalfa (*Medicago sativa* L.). *Chemosphere,* 71:1593-1598. https://doi.org/10.1016/j.chemosphere.2007.10.068

Genot, V., Colinet, G., Brahy, V., Bock, L., 2009. L’état de fertilité des terres agricoles et forestières en région wallonne (adapté du chapitre 4 - sol 1 de « L’État de l’Environnement wallon 2006-2007 »). *Biotechnol Agron Soc Environ.,* 13(1): 121–138.

Ghosal, D., Ghosh, S., Dutta, T.K., Ahn, Y., 2016. Current state of knowledge in microbial degradation of polycyclic aromatic hydrocarbons (PAHs): A review. *Front Microbiol.,* 7:1369. https://doi.org/10.3389/fmicb.2016.01369

Hall, J., Soole, K., & Bentham, R., 2011. Hydrocarbon phytoremediation in the family *Fabaceae*-a review. *Int J Phytoremediation,* 13:317–332. https://doi.org/10.1080/15226514.2010.495143

Hamdi, H., Benzarti, S., Aoyama, I., Jedidi, N., 2012. Rehabilitation of degraded soils containing aged PAHs based on phytoremediation with alfalfa (*Medicago sativa* L.). *Int Biodeterior Biodegrad.,* 67:40-47. https://doi.org/10.1016/j.ibiod.2011.10.009

Henner, P., Schiavon, M., Druelle, V., Lichtfouse, E., 1999. Phytotoxicity of ancient gaswork soils. Effect of polycyclic aromatic hydrocarbons (PAHs) on plant germination. *Org Geochem.,* 30:963-969. https://doi.org/10.1016/S0146-6380(99)00080-7

Institut National de l’Environnement Industriel et des Risques (INERIS), 2005. Hydrocarbures Aromatiques Polycyliques - Guide méthodologique - Acquisition des données d’entrée des modèles analytiques ou numériques de transferts dans les sols et les eaux souterraines. https://doi.org/10.1017/CBO9781107415324.004

ISO 11465:1993 cor 1994. Soil quality - Determination of dry matter and water content on a mass basis - Gravimetric method.

ISO 13877:1998. Soil quality - Determination of polynuclear aromatic hydrocarbons - Method using high-performance liquid chromatography.

ISO/TS 16751:2018. Soil quality - Environmental availability of non-polar organic compounds - Determination of the potential bioavailable fraction and the non-bioavailable fraction using a strong adsorbent or complexing agent.

Johnsen, A.R., Wick, L.Y., & Harms, H., 2005. Principles of microbial PAH-degradation in soil. *Environ Pollut.,* 133:71-84. https://doi.org/10.1016/j.envpol.2004.04.015

Kang, F., Chen, D., Gao, Y., Zhang, Y., 2010. Distribution of polycyclic aromatic hydrocarbons in subcellular root tissues of ryegrass (Lolium multiflorum Lam.). *BMC Plant Biol.,* 10(210). https://doi.org/10.1186/1471-2229-10-210

Kass, R.E., & Raftery, A.E., 1995. Bayes Factors. *J Am Stat Assoc.,* 90(430): 773–795.

Keith, L.H., 2015. The Source of U.S. EPA’s Sixteen PAH Priority Pollutants. *Polycycl Aromat Comp.,* 35:147–160. https://doi.org/10.1080/10406638.2014.892886

Laha, S., Tansel, B., & Ussawarujikulchai, A., 2009. Surfactant-soil interactions during surfactant-amended remediation of contaminated soils by hydrophobic organic compounds: A review. *J Environ Manage.,* 90:95-100. https://doi.org/10.1016/j.jenvman.2008.08.006

Leigh, M.B., Fletcher, J.S., Fu, X., Schmitz, F.J., 2002. Root turnover: An important source of microbial substrates in rhizosphere remediation of recalcitrant contaminants. *Environ Sci Technol.,* 36:1579-1583. https://doi.org/10.1021/es015702i

Martin, B.C., George, S.J., Price, C.A., Ryan, M.H., Tibbett, M., 2014. The role of root exuded low molecular weight organic anions in facilitating petroleum hydrocarbon degradation: Current knowledge and future directions. *Sci Total Environ.,* 472:642-653. https://doi.org/10.1016/j.scitotenv.2013.11.050

Medina, R., Fernández-González, A.J., García-Rodríguez, F.M., Villadas, P.J., Rosso, J.A., Fernández-López, M., Del Panno, M.T., 2020. Exploring the effect of composting technologies on the recovery of hydrocarbon contaminated soil post chemical oxidative treatment. *Appl Soil Ecol.,* 150: 103459. https://doi.org/10.1016/j.apsoil.2019.103459

Müntz, K., Belozersky, M.A., Dunaevsky, Y.E., Schlereth, A., Tiedemann, J., 2001. Stored proteinases and the initiation of storage protein mobilization in seeds during germination and seedling growth. *J Exp Bot.,* 52(362):1741-1752.

Nzila, A., 2018. Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons under anaerobic conditions: Overview of studies, proposed pathways and future perspectives. *Environ Pollut.,* 239:788-802. https://doi.org/10.1016/j.envpol.2018.04.074

Oleszek, W., & Bialy, Z., 2006. Chromatographic determination of plant saponins-An update (2002-2005). *J Chromatogr A,* 1112:78-91. https://doi.org/10.1016/j.chroma.2006.01.037

Olson, P.E., Castro, A., Joern, M., Duteau, N.M., Pilon-Smits, E.A.H., Reardon, K.F., 2007. Comparison of plant families in a greenhouse phytoremediation study on an aged polycyclic aromatic hydrocarbon-contaminated soil. *J Environ Qual.,* 186:1271-1276. https://doi.org/10.2134/jeq2006.0371

Ouvrard, S., Leglize, P., & Morel, J.L., 2014. PAH Phytoremediation: Rhizodegradation or Rhizoattenuation? *Int J Phytoremediation,* 16(1): 46-61. https://doi.org/10.1080/15226514.2012.759527

Posada-Baquero, R., López Martín, M., & Ortega-Calvo, J.J., 2019. Implementing standardized desorption extraction into bioavailability-oriented bioremediation of PAH-polluted soils. *Sc Total Environ.,* 696:134011. https://doi.org/10.1016/j.scitotenv.2019.134011

Posada-Baquero, R., Jiménez-Volkerink, S.N., Luis García, J., Vila, J., Cantos, M., Grifoll, M., Ortega-Calvo, J.J., 2020. Rhizosphere-enhanced biosurfactant action on slowly desorbing PAHs in contaminated soil. *Sc Total Environ.,* 720:137608. https://doi.org/10.1016/j.scitotenv.2020.137608

Prague, M., Diakite, A., & Commenges, D., 2012. Package ' marqLevAlg ' - Algorithme de Levenberg-Marquardt en R : Une alternative à ' optimx ' pour des problèmes de minimisation. HAL Id : hal-00717566.

Reichenauer, T.G., & Germida, J.J., 2008. Phytoremediation of organic contaminants in soil and groundwater. *ChemSusChem*, 1:708-717. https://doi.org/10.1002/cssc.200800125

Schwab, A.P., Al-Assi, A.A., & Banks, M.K., 1998. Adsorption of Naphthalene onto Plant Roots. *J Environ Qual.*, 27(1):220-224. https://doi.org/10.2134/jeq1998.00472425002700010031x

Semple, K.T., Morriss, A.W.J., & Paton, G.I., 2003. Bioavailability of hydrophobic organic contaminants in soils: fundamental concepts and techniques for analysis. *Eur J Soil Sci.*, 54:809-818.

Semple, K.T., Doick, K.J., Jones, K.C., Burauel, P., Craven, A., Harms, H., 2004. Peer Reviewed: Defining Bioavailability and Bioaccessibility of Contaminated Soil and Sediment is Complicated. *Environ Sci Technol.*, 229-231. https://doi.org/10.1021/es040548w

Smith, M.J., Flowers, T.H., Duncan, H.J., Alder J., 2006. Effects of polycyclic aromatic hydrocarbons on germination and subsequent growth of grasses and legumes in freshly contaminated soil and soil with aged PAHs residues. *Environ Pollut.*, 141:519-525. https://doi.org/10.1016/j.envpol.2005.08.061

Sparg, S.G., Light, M.E., & Van Staden, J., 2004. Biological activities and distribution of plant saponins. *J Ethnopharmacol.*, 94:219-243. https://doi.org/10.1016/j.jep.2004.05.016

Taiz, L., Zeiger, E. *Plant Physiology*. Sunderland, MA: Sinauer.

Sverdrup, L.E., Krogh, P.H., Nielsen, T., Kjær, C., Stenersen, J., 2003. Toxicity of eight polycyclic aromatic compounds to red clover (*Trifolium pratense*), ryegrass (*Lolium perenne*), and mustard (*Sinapsis alba*). *Chemosphere*, 53:993-1003. https://doi.org/10.1016/S0045-6535(03)00584-8

Teng, Y., Shen, Y., Luo, Y., Sun, X., Sun, M., Fu, D., Li, Z., Christie, P., 2011. Influence of Rhizobium meliloti on phytoremediation of polycyclic aromatic hydrocarbons by alfalfa in an aged contaminated soil. *J Hazard Mater.*, https://doi.org/10.1016/j.jhazmat.2010.11.126

Zhou, W., Yang, J., Lou, L., Zhu, L., 2011. Solubilization properties of polycyclic aromatic hydrocarbons by saponin, a plant-derived biosurfactant. *Environ. Pollut.*, 159:1198-1204. https://doi.org/10.1016/j.envpol.2011.02.001

9. Supplementary material

Several models were tested to describe the PAHs desorption kinetics. BIC values were calculated according to equation (5) to determine the best model for each PAH desorption dataset.

$BIC = k.\ln(n) - 2.ln(L)$

with k the number of parameters of a model, n the number of data points and L the maximized value of a likelihood function. R function is BIC(model_iner2).

Supplementary table 4. Several models were tested to describe the PAH desorption kinetics. The Bayesian information criterion (BIC) values were calculated as follows to determine the best model for each PAH desorption dataset: $BIC = k.\ln(n) - 2.ln(L)$ with k the number of parameters of a model, n the number of data points and L the maximized value of a likelihood function. The R function is BIC (model_iner2).

	Model			
PAHs	**Firt-order**	**Firt-order**	**Firt-order**	**Site distribution**
		Two-Compartments	**Three-Compartments**	
N	-268	-273	-266	-278*
Ace	-233	-255	-248	-261*
Fle	-342	-363	-368*	-365
Phen	-355	-370	-371	-372*
Anthr	-362	-390	-400*	-397
F	-373	-388	-390	-390*
Pyr	-419	-439	-443	-445*
BaA	-354	-377	-382	-388*
Chrys	-339	-380*	-357	-377
BbF	-340	-360	-352	-376*
BkF	-341	-369	-378	-386*
BaP	-354	-378	-392	-397*
DBahA	-329	-351	-342	-370*
BghiP	-318	-360	-335	-365*
IcdP	-338	-353	-356	-369*
∑2-3 rings	-365	-380	-382*	-378
∑4 rings	-392	-410	-412	-412*
∑4-6 rings	-342	-365	-371	-383*
∑all	-357	-381	-386	-394*

* smallest BIC values for each PAH.

Part 4

Targeting the right parameters in PAHs remediation studies

1. Foreword

The thesis was built over a few assumptions gathered during the pre-thesis literature research. For example, the assumption that PAHs contents lowered in soils in presence of some vascular plants has already been faced with contradictory results exposed previously. Another assumption was the fact that soil remediation has to aim for the maximal possible dissipation of pollution, or the fact that they are only 16 PAHs of interest that need to be remediated. But this work has been a long learning process along which a few reflections were made concerning research on PAHs remediation in aged-contaminated soil. Therefore, three scientific trends that currently lead PAHs contaminated soils/sediments remediation studies and management, as well as future orientations this area of research should consider leaning towards are being discussed hereafter. The following topics have been discussed in a critical review that was submitted for publication: (i) the choice of compounds that are being studied and targeted in scientific literature, (ii) the choice of experimental material in remediation studies (i.e. freshly contaminated or co-contaminated and aged-contaminated material), and (iii) the systematic use of the recently validated bioavailability measurement protocol (ISO/TS 16751:2018) in remediation trials.

2. Abstract

Contaminated lands burden the economy of many countries and must be dealt with.

Searchers have published thousands of documents studying and developing soil and sediment remediation treatments. Amongst the targeted pollutants are the polycyclic aromatic hydrocarbons (PAHs), described as a class of persistent organic compounds, potentially damageable to ecosystems and living organisms.

The present paper reviews and discusses three scientific trends that lead PAHs contaminated soils/sediments remediation studies and management.

Firstly, the choice of compounds that are being studied and targeted in scientific literature is discussed, as we suggest that the classical 16 US-EPA PAHs compounds might no longer be sufficient to meet actual environmental challenges.

Secondly, we discuss the choice of experimental material in remediation studies. Using bibliometric measures, we show the lack of PAHs remediation trials based on co-contaminated or aged-contaminated material.

Finally, the systematic use of the recently validated bioavailability measurement protocol (ISO/TS 16751:2018) in remediation trials is discussed, as we suggest it should be implemented as a tool to improve remediation processes and management strategies.

3. Introduction

Countries which are or have been heavily industrialised own their share of brownfields that often present multiple types and levels of contaminations. Brownfields are a legacy which burden this generation, and probably many more to come, and have to be dealt with. First because any unmanaged contamination is a potential threat towards the environment at large, but also because the majority of these sites are no longer appropriate to host any type of activity (agricultural, residential, nor industrial) as long as they have not been remediated, and this constitutes a huge economic loss. At a time when the world's population is growing fast, a sustainable use of natural resources is crucial to meet the United Nation "Sustainable Development Goals" (Umeh *et al.,* 2017).

The objectives of a review are to highlight new progress, successes and sometimes failures. But most importantly a review should point new directions or areas that lack data or knowledge. It is also the objective of this paper, which aims at questioning scientific approaches that have been leading contaminated soil remediation studies and management, and more specifically polycyclic aromatic hydrocarbons (PAHs) remediation in soils/sediments. Firstly, for the past decades, worldwide scientific publications have focussed on studying a rather short list of PAHs, namely the 16 PAHs from the American Environmental Protection Agency's (US-EPA) "Priority Pollutants" list published in 1978 (Keith, 2015), seemingly without ever questioning its content. Secondly, PAHs remediation techniques have been developed for several decades, with the underlying goal being to provide solutions to eliminate pollutions from actually contaminated environment compartments. Yet, when performing a bibliometric analysis of all types of documents that have been published and researches that have been led, it is striking to realize that only a small fraction of the publications on the matter actually concentrates on realistic aged-contaminated soils, not to mention the lack of studies focussing on multiple contaminations. Finally, PAHs remediation endpoints will be discussed. When it comes to environmental regulations and soil remediation guidelines, the driving assumption is that (aged)-contaminated soils must be remediated to the greater possible extent. It has recently been pointed out in several reviews that there is a need to implement a risk-based approach using a bioavailability parameter to establish site management and decontamination strategies. But we suggest that this bioavailability parameter be taken further and used when developing remediation treatments, as it would bring valuable insight on the processes at place.

4. On the use of the 16 “Priority Pollutants” PAHs

The study of contaminations as (potential) threats to the environment and human populations gave birth to thousands of scientific publications on the subject. Pollutants are traditionally separated between inorganic and organic pollutants. The list of inorganic pollutants is rather well-defined, as it comprises a series of trace metals and metalloids often referred to as “heavy metals” (Duffus, 2002). But the list of organic pollutants is made of dozens of groups (e.g. PAHs, PCBs, PCDDs, PCDFs, BTEX, …) and new pollutants are still being pointed out by scientists, as potentially harmful effects are highlighted by research everyday (e.g. pharmaceutical products) (Reichert *et al.,* 2019). Besides, each group of organic pollutants often contains a large variety of compounds. For instance, PAHs are commonly defined as molecules made of two or more condensed aromatic rings placed in linear, angular or clustered arrangements (Dhar *et al.,* 2019). When encountered in soil or sediments, they are of two main origins: petrogenic (which usually implies that products of petroleum origin were spilled) and pyrogenic (meaning compounds are created during incomplete combustions) (Iqbal *et al.,* 2008; Dhar *et al.,* 2019). PAHs contaminations can be of natural causes (e.g. volcanic episodes or forest fires) but anthropogenic activities are mostly to blame (e.g. fuel combustion, waste incineration or accidental spill) (Nzila, 2018). In the scientific literature, this PAHs definition is commonly followed by the same list of 16 PAHs compounds. It is however rarely mentioned that the list was established over forty years ago, under time pressure, and needs to be re-assessed according to the knowledge that has been acquired for the past decades and to today’s environmental management challenges. The classical PAHs watch list was established in 1976 by the US-EPA, when a general awakening took place with regards to the issue of water organic pollution. Among other classes of pollutants, the US-EPA selected 16 PAHs as “Priority Pollutants”. These PAHs made it to the list mainly because (i) they had previously been detected in several water contamination reports on North-American land (>5%) and (ii) they were commercially available so that a standard could be used to confirm identification in analytical methods (Keith, 2015). The original list only contained specific isomers and apolar PAHs because at the time, the reference analytical instrument (gas chromatography coupled to mass spectrometry) was not reliable to detect isomers, and commercial alkylated PAHs were difficult to find (Keith, 2015). Afterwards, this list served as a consistent basis for scientific research, results comparison (Andersson & Achten, 2015) and for other countries to establish environmental regulation guidelines (Keith, 2015). However, this list has not evolved with regards to the PAHs compounds ever since. But in forty years, health and environmental challenges have evolved, major knowledge was acquired, and analytical methods were developed.

There are more than 16 compounds to be concerned of, and it is interesting to notice the slight offset between the commonly cited 16 apolar PAHs in scientific research and the compounds present in legislations or international scientific committees’ reports. Not all countries in the world are yet equipped with soil quality guidelines, but some do have other regulations that present with hazardous substances watch lists.

For example in the European Union, there is still no Soil Protection Framework Directive, but there is a Water Framework Directive (WFD) (EC, 2020), a Food Regulation (EFSA, 2020), a Chemicals (REACH) Regulation and even a Persistent Organic Pollutants (POPs) Regulation (ECHA, 2020) which all take aim at the protection of human health and environment. On a broader scale, Canada is equipped with Soil Quality Guidelines (CCME, 2020) and the World Health Organization, though it does not provide soil quality guidelines, had experts work on an international programme on chemical safety and establish environmental health criteria (WHO, 2020). When comparing the polycyclic aromatic compounds mentioned in these regulations or watch lists (all available in Supplementary table 5) and the US-EPA list on which most soil remediation studies lean, one can notice a few discrepancies. For instance (Table 13), the WHO mentions 17 compounds besides the usual 16, among which 15 are apolar compounds and several are isomers of compounds mentioned in the US-EPA list (e.g. benzo[j]fluoranthene and benzo[k]fluoranthene). Another example is the European food regulation list which mentions 15 compounds, all of which are mentioned on the WHO list, but out of which only 8 compounds are common to the US-EPA list.

Table 13. Comparison of the polycyclic aromatic compounds of the US-EPA watch list (EPA, 2020) to the compounds present in the WHO (WHO, 2020) and the European Union Food Regulation (EFSA, 2020) watch lists. Compounds in bold are from the US-EPA watch list.

Compound	**Watch list**	
	WHO	**European Union Food Regulation**
1-methylphenanthrene	x	
5-methylchrysene	x	x
Acenaphthene	x	
Acenaphtylene	x	
Anthanthrene	x	
Anthracene	x	
Benzo[a]anthracene	x	x
Benzo[a]fluorene	x	
Benzo[a]pyrene	x	x
Benzo[b]fluoranthene	x	x
Benzo[b]fluorene	x	
Benzo[c]phenanthrene	x	
Benzo[e]pyrene	x	
Benzo[ghi]fluoranthene	x	
Benzo[ghi]perylene	x	x
Benzo[j]fluoranthene	x	x

(continued)

Compound	**Watch list**	
	WHO	**European Union Food Regulation**
Benzo[k]fluoranthene	x	x
Chrysene	x	x
Coronene	x	
Cyclopenta[c,d]pyrene	x	x
Dibenz[a,h]anthracene	x	x
Dibenzo(a,e)pyrene	x	x
Dibenzo(a,h)pyrene	x	x
Dibenzo(a,i)pyrene	x	x
Dibenzo(a,l)pyrene	x	x
Fluoranthene	x	
Fluorene	x	
Indeno[1,2,3-cd]pyrene	x	x
Naphthalene	x	
Perylene	x	
Phenanthrene	x	
Pyrene	x	
Triphenylene	x	

PAHs are part of a larger group of polycyclic aromatic compounds (PACs) that are not always apolar and can contain heteroatoms such as oxygen, nitrogen, sulphur… (Bowman *et al.,* 2019). PAHs themselves can be substituted with halogens, alkyl-, oxy-, hydroxyl-, amino- or nitro-functional groups, and then there is the matter of NSO-heterocycles, which are aromatic rings containing nitrogen, sulphur or oxygen (Andersson & Achten, 2015). A very complete review on the matter of substituted and heteroatomic PACs' origin, properties and fate in the environment was published by Idowu *et al.,* (2019) who insisted on the fact that such compounds are less studied than apolar PAHs. However, it is crucial that the scientific community and the legislators start taking these different types of PACs seriously. (i) Because many of those compounds are believed to be more genotoxic, mutagenic and carcinogenic than apolar PAHs (Bleeker *et al.,* 1999; Park *et al.,* 2008; Lundstedt *et al.,* 2014; Andersson & Achten, 2015; Tian *et al.,* 2017a). (ii) Because heteroatomic PACs are more polar, and therefore suspected to be more mobile in the environment (Bowman *et al.,* 2019). (iii) Because depending on their origin, some of these compounds are present along with apolar PAHs (Idowu *et al.,* 2019). Most PAHs of petrogenic origin are of low molecular weight (two or three rings) and they also contain a majority of alkylated PAHs. However PAHs of pyrogenic origin are dominated by unsubstituted compounds of high molecular weight (four, five, six rings) (Bowman *et al.,* 2019; Iqbal *et al.,* 2008). But no matter their origin, PAHs can occur with PACs as co-contaminants (Tian *et al.,* 2017b; Idowu *et al.,* 2019). And (iv) because alkylated and heteroatomic PACs can appear through secondary processes of apolar PAHs, such as (photo)chemical degradation and biological degradation (Lundstedt *et al.,* 2002; Hu *et al.,* 2012; Chibwe *et al.,* 2017; Tian *et al.,* 2017a; Idowu *et al.,* 2019).

The fact that substituted PAHs can be metabolites from the incomplete degradation of apolar PAHs should cast some questioning towards the way remediation strategies such as bioremediation are being led. Bioremediation relies on microbial biodegradation to mineralize PAHs, which takes place naturally in the environment. This is why metabolic pathways, especially the bacterial aerobic ones, have been intensively studied for decades (Ghosal *et al.,* 2016). The 16 US-EPA PAHs are the usual targets of all these studies and, as several apolar PAHs degradation pathways are now established, it is well-known that mineralization processes can meet dead-ends (Idowu *et al.,* 2019). When present in mixtures, phenomena of augmentation, cometabolism, or inhibition can influence both the extent and rate of individual PAHs degradation, depending on the type of mixture but also the degrading microbial consortia (Mahanty *et al.,* 2011). Those enhancing or inhibiting phenomena were highlighted by studies conducted in controlled conditions, implying a few PAHs (pure or in mixtures) and a few specific strains (in individual or mixed cultures) (Bouchez *et al.,* 1995; Stringfellow & Aitken, 1995). But these phenomena are not systematically encountered and are difficult to predict. PAHs encountered in a polluted environment are present in mixtures. But microbial communities are also much more diverse than can be accounted for in controlled culture studies, and it is thus likely that in presence of mixed microbial species, degradative pathways complete each other and intermediate or dead-end metabolites can be substrates for other species (Mahanty *et al.,* 2011; Vila *et al.,* 2015). But when metabolites such as epoxides, quinones, ketones or hydroxylated-PAHs are left in the soil instead of reaching complete mineralization, it must raise concern because such compounds may be more toxic than their parent PAH (Ghosal *et al.,* 2016; Davie-Martin *et al.,* 2017; Chibwe *et al.,* 2017). Indeed, some studies have used bioassays to highlight the fact that though bioremediation treatments might lower the content of apolar PAHs, the general (geno)toxicity or mutagenicity of the treated soil could increase during the process (Hu *et al.,* 2012; Chibwe *et al.,* 2015). What is even more concerning is that the presence of toxic metabolites is not systematically monitored. Indeed, when soils are being remediated, the final remediation goals, whether in scientific studies or in realistic aged-contaminated soil remediation, are expressed as the lowering of the initial apolar PAHs contents that must be reached. But knowing that complete PAHs degradation is difficult to achieve, and to predict, maybe it is time to consider adding the monitoring of transformation metabolites to the management of polluted soil, especially when remediation techniques are applied.

Fortunately, these topics have been at the centre of several research papers over the last few years. Besides showing that a soil's toxicity may increase during remediation, a few studies have focussed on the isolation, purification, and identification of the metabolites responsible for this enhanced toxicity (e.g. Chibwe *et al.,* 2017; Tian *et al.,* 2017a) and very often, oxygen-containing metabolites were pointed out. Also, analytical methods have been under development to detect nitro-PAHs, oxy-PAHs, hydroxy-PAHs, methyl-PAHs, halogenated-PAHs, or even N-heterocycles, sometimes along with apolar PAHs (Niederer, 1998; Cochran *et al.,* 2012; García-Alonso *et al.,* 2012; Tian *et al.,* 2017a; Mueller *et al.,* 2019; Bowman *et al.,* 2019; Wickrama-Arachchige *et al.,* 2020). Unlike for the determination of the 16 US-EPA PAHs, the analytical methods are diverse, and some have not yet reached complete quantification. Attempts are being made to harmonize the methods (as was the case for some oxy-PAHs and N-heterocycles in the intercomparison study led by Lundstedt *et al.,* (2014)), but the work is highly complicated by the fact that there is still a lack of consensus concerning the compounds that should be analysed, as well as a lack of reference materials. Of course, there are so many possible metabolites that it is impossible to monitor every by-product during remediation processes. But since analytical methods are being developed and awareness on the matter is being risen, it really is worth, from a risk-analysis point-of-view, starting to look for different types of polycyclic aromatic compounds, and include some of them in watch lists.

These few examples show a lag between regulations and research and highlight the fact that the scientific community should broaden the list of studied polycyclic aromatic compounds, not only in the matter of soil remediation studies but also in land management and environmental risk-assessment. First, it would address environmental challenges faced by countries (and their regulations) with different hazardous pollutants watch lists, and second, it might highlight remediation or naturally occurring dead-ends and could bring new perspective to land management strategies at large. Andersson & Achten, (2015) initiated this reflexion as they suggested, based on toxicity, occurrence, and ease of analysis, to enlarge the classical list of 16 US-EPA PAHs by adding 24 compounds (alkylated and apolar PAHs) for environmental toxicity evaluation. They also suggested 23 NSO-heterocyclic compounds, 6 heterocyclic metabolites, 10 oxy-PAHs, and 10 nitro-PAHs that would be of interest to monitor in the future. But as mentioned previously, analytical methods are improving and progress is still being made to identify toxic metabolites, meaning reflexion and research are still necessary on this matter.

5. On the use of realistic aged-contaminated soil in research

During the past three decades, the development of PAHs remediation techniques in soils/sediments started to show diversity. Research tends to evolve quickly and to spread in many directions, and it is useful, once in a while, to establish the state-of-the-art of a topic. To make the work sustainable, it is often necessary to narrow the topic to a few specific items. For example in the matter of PAHs soils/sediments remediation, reviews describing recent advances in remediation techniques have focussed on certain categories of treatments, such as the electroremediation of PAHs (Pazos *et al.,* 2010), the extraction agents used for PAHs soil washing (Von Lau *et al.,* 2014), the surfactant-enhanced remediation of PAHs (Lamichhane *et al.,* 2017), or the microbe-enhanced phytoremediation of PAHs (Sarma *et al.,* 2019). This section of the paper focusses on the fact that the long-term objective of researches on remediation treatments is to develop techniques to treat PAHs-contaminated soils/sediments of all ages and types. Indeed, the ultimate goal is to bring solutions to the management and remediation of contaminated land. Published techniques can be more or less efficient, cost-effective, or environmentally-friendly, but they all are being led under that same banner, since they all start by exposing the need for PAHs remediation due to their potential or confirmed toxicity. However, when examined as a whole, they sometimes seem to be slightly out of focus.

Several databases were explored to highlight published documents that actually studied aged-contaminated soils/sediments and tested techniques on realistic matrices, with all their complexity. The point was not to dissect every single study and its outcome, but to question whether the scientific community takes the testing of remediation treatments as far as it can, or should. Therefore, bibliometric tools were used. All details, including data, are available in supplementary material. Please note that for the sake of clarity, single terms representing groups of searching terms are used (e.g. “aged” states for “aged or ancient or former or historical”), and a few representative treatments are discussed that aim to cover as much of the diversity of remediation publications as possible.

The first three searches narrowed down the number of published documents (1) on PAHs in general, (2) on PAHs in soils or sediments, and (3) on the remediation of PAHs in soils or in sediments (Table 14). Out of 2901 to 87248 documents related to PAHs (1), depending on the databases, 1156 to 28789 documents focus on PAHs in soils or in sediments (2), which represents an average 31 ± 4 % of the global PAHs documents (1). Also, 260 to 6267 documents focus on PAHs remediation in soils or in sediments (3), representing respectively 7 ± 1 % and 22 ± 1 % of the global PAHs documents (1) and of the PAHs in soils/sediments documents (2) (see Supplementary table 6 for detailed calculations).

Two others searches were conducted to highlight the documents that focused (4) on soils or sediments presenting multiple types of contaminations, and (5) on aged soils or sediments with multiple contaminations, both on PAHs remediation (3) (Table 14). The highest results are respectively 0.23 % and 0 % (Supplementary table 6) and clearly show the lack of attention that has been brought to the matter of multiple contaminations in the area of PAHs soils/sediments remediation yet, even though most contaminated areas present with multiple types of contaminations (Deary *et al.*, 2018). This does not necessarily mean that studies are not being conducted on soils/sediments presenting multiple contaminations, but more probably that research in general has not moved yet on trying to remediate several types of contaminations at a time. An interesting example of a phytoremediation trial assisted by the addition of a complexing agent on soil co-contaminated with cadmium and fluorene was published by Wang et al. (2018). The soil was spiked with the pollutants prior trial, but the study shows interest in multiple contaminants clean-up strategies.

Publications concerning PAHs remediation in soils/sediments (3) were narrowed down to several categories of treatments (heating, electrokinetic or electrochemical, washing, solubilisation, chemical oxidation, bioremediation and phytoremediation), then narrowed again to highlight the aged character of the pollution. The proportions of documents focussing on aged experimental material within each category of treatments, as well as in remediation documents in general (3) were then calculated (Supplementary table 7). The average proportions are displayed in Figure 19. Values range from 7 ± 1 % for electrokinetic and electrochemical treatments to 33 ± 11 % for heating treatments. Concerning documents on PAHs remediation in general, the average proportion is 12 ± 0.4 %. This, combined to the very low number of documents related to multiple contaminations, shows that the scientific community is not working on realistic soils/sediments on a regular basis yet.

When developing a remediation process, scientists try to understand the mechanisms that rule it, which is why very often preliminary studies tend to focus on one, then a few representative PAHs at a time, and to work in simplified controlled conditions. So, it is common to start working in aqueous media, for example to study biodegradation mechanisms, and then move on to freshly spiked soil. But whilst working with simplified models brings very valuable information and is always the best way to screen the potential of an innovative technique, it is unfortunately not representative of the reality of aged-contaminated soils which are to be dealt with. Indeed, most contaminated lands display multiple contaminations, of either organic or inorganic nature, which have been in place for decades and have partitioned, sometimes very deeply, into the soil compartment. There were enough published studies on that matter to acknowledge that these ageing processes complicate greatly the remediation, especially when the long-term objectives are to remove the pollution to the greater possible extent, and to bring pollutant contents down.

The question is, why is there, apparently, still such little work being made on realistic soils/sediments? Is it because scientists tend to lose sight of their final objective, i.e. the remediation of realistic contaminated land? Is it because there is still a lack of knowledge that should be acquired by working in controlled experimental conditions before actually moving on to realistic conditions?

Table 14. Number of documents published in English on PAHs (1), on PAHs in soils or sediments (2), on the remediation of PAHs in soils or sediments (3), on the remediation of PAHs in soils or sediments presenting multiple contaminations (4), and on the remediation of PAHs in aged soils or sediments presenting multiple contaminations (5) in a series of databases until the end of year 2019.

Number of documents	**Question number**	1	2	3	4	5
	Searching terms	PAHs				
			soils or sediments			
				remediation		
					multiple contaminations	
						aged
Database						
AGRICOLA		17613	5735	1404	0	0
Agricu. & Environ. Science Collection		87248	28789	6267	2	0
Agricu. Science Collection		20514	6891	1661	0	0
Agriculture Science Database		2901	1156	260	0	0
ASP		47249	6150	1407	2	0
CAB ABST		29566	10886	2629	4	0
Environment Complete		24177	8650	1992	4	0
Environmental Science Collection		68228	22674	4738	2	0
Environmental Science Database		9781	3574	795	0	0
Environmental Science Index		66508	22466	4692	2	0
GreenFILE		11936	4648	917	1	0
Medline		20474	4963	884	2	0
Scopus		70111	13368	3283	6	0
TOXLINE		15765	4044	732	0	0

Or worse, is it because the results of experiments on realistic soils/sediments are so negative or inconclusive that they are not being shared for common knowledge? Technical difficulties in leading reproductive and representative experiments on realistic soil samples are probably the main issue. Indeed, pollution is rarely, if not never, present in a homogeneous way in the environment. Research should be as reproducible as possible and thus requires to work on homogenous material, meaning manipulations such as sieving and mixing are often necessary. This *de facto* will render the experimental material less representative than the state it was originally in. Also, two experimental materials, no matter how similar in physico-chemical properties (particle distribution, moisture, compaction, oxygenation, but also types and levels of contaminations), will never be exactly the same, making conclusions on one specific realistic material difficult to generalize. A simple example is the variety of source materials through which PAHs can be brought and released into the soil compartment. Whether PAHs are brought in through non-aqueous phase liquids (NAPLs, such as gasoline) or solids (such as coke) will influence the release and sorption of PAHs in soil/sediment (Yu *et al.*, 2018), even if those different source materials might lead to similar levels of PAHs contamination. Finally, and as mentioned previously, the source and origin of PAHs (e.g. pyrogenic or pyrolytic) will bring different types of co-contaminants (e.g. other PACs, but also heavy metals, other organic pollutants such as PCBs, BTEX…). In an ideal research scenario, complete knowledge of the experimental material levels and types of contaminations would be necessary to gather as much information on the remediation processes and interactions at stake. But the variety of contaminants present in realistic soils/sediments renders exhaustive characterization extremely difficult (if not impossible) and expensive. A good start would be to narrow down this characterization to a few main groups of contaminants and to examine the effects co-contaminants and remediation techniques have on each other.

Nevertheless, as challenging as working on more realistic material might be, it should not be postponed because it is too complex. It is crucial that, once research has given encouraging results in controlled conditions, the potential new treatment is brought to the next level: the testing on realistic soils/sediments, and preferably a variety of them. And if the next level is inconclusive or somewhat disappointing, it is still important to publish these outcomes so that other searchers can try and improve the treatment, and not waste time on repeating the same experiment which will likely be considered a failure too. After all, that’s what science is based on: sharing knowledge.

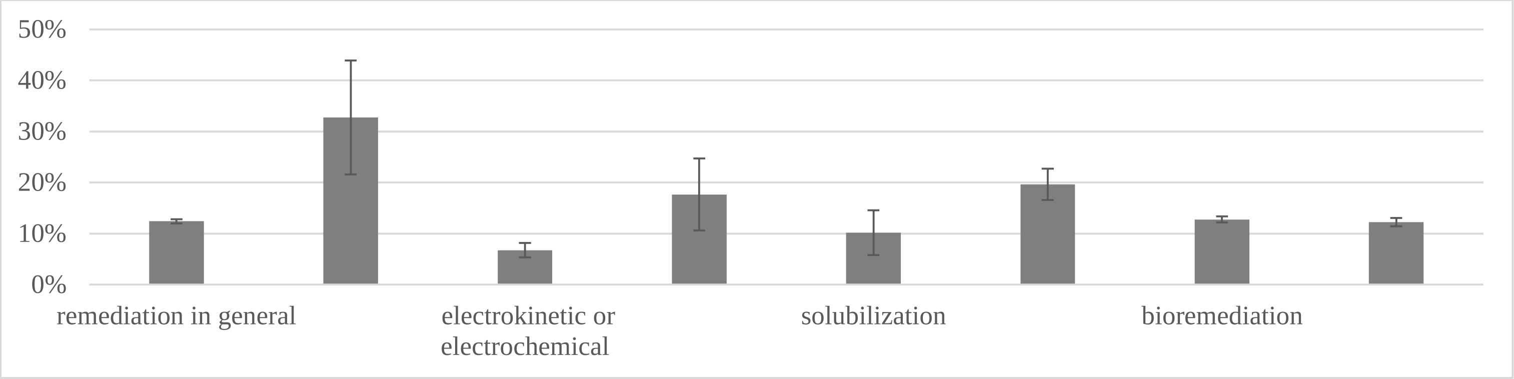


Figure 19. Proportions of documents on aged soils/sediments in several clusters of documents on PAHs remediation treatments. Values are means ± confidence intervals (α=0.05). See Supplementary table 7 for detailed numbers and calculations.

6. On the use of the bioaccessibility parameter in remediation studies

Three decades ago, the scientific community started to focus on the concept of PAHs' bioavailability. Searchers were gathering encouraging results and increasing knowledge regarding PAHs metabolism (mainly under aerobic conditions) in the laboratory, but failed to predict outcomes in field conditions (Sanseverino *et al.*, 1993). They were facing poor PAHs mineralization rates and yields even in presence of favourable conditions. Research and publications focussed on several aspects of bioavailability: (i) defining it, (ii) identifying the factors that influence it, (iii) measuring it, and (iv) increasing it towards degrading microorganisms (in the context of remediation).

Settling on concepts and definitions alone has been at the centre of many publications and reviews (Ehlers & Luthy, 2003; Semple *et al.*, 2003; Semple *et al.*, 2004; Reichenberg & Mayer, 2006; Semple *et al.*, 2007; Ortega-Calvo *et al.*, 2015). Concepts as crucial as "chemical activity", "bioavailability", "bioaccessibility", "non-extractable residues" (NERs) and the processes that govern them were defined, and will not be repeated here. Please note that the term "bioavailability" is used as a generic term.

The factors and the sorption/desorption mechanisms influencing organic compounds' bioavailability (including PAHs) have been, and still are, thoroughly investigated and reviewed. They include (i) soils/sediments properties such as solid and dissolved organic matter (SOM and DOM) content, particle size, chemical structure, composition, polarity, mineral composition or organo-mineral associations; (ii) environmental factors (pH, temperature, moisture…); (iii) characteristics of the contamination such as the source material (atmospheric emission, solid, semi-solid, (NAPLs)), the presence of co-contaminants, or the initial amounts of pollutants, and (iv) microbial capacities such as the type and variety of degrading species, their morphological, behavioural, physiological adaptations, and chemotactic capabilities (Ortega-Calvo *et al.,* 2013; Duan *et al.,* 2015; Ren *et al.,* 2018; Yu *et al.,* 2018).

Several methods to measure bioavailability have been developed, and reviewed as well (Semple *et al.,* 2007; Cui *et al.,* 2013; Riding *et al.,* 2013; Cachada *et al.,* 2014). Recently, the ISO/TS 16751:2018 norm settled some debates on bioavailability measurement by defining a protocol using either a strong sorbent (Tenax®) or complexing agents (cyclodextrins) to determine the "bioavailable fraction" of non-polar organic compounds (such as PAHs), also named "environmental availability". The norm uses biomimetic surrogates, which are meant to imitate a potential maximal uptake from the aqueous solution by organisms. This environmental availability is defined in norm ISO 17402 (2008) as "the fraction of a contaminant actually or potentially available to organisms", which is the definition of bioaccessibility by Semple et al. (2004). It is different from the environmental bioavailability, which includes uptake by the organisms and is dependent on the biological group or even the species. Indeed, aqueous diffusion (on which relies a biomimetic method to measure the environmental availability) is not the only mechanism through which organisms might be exposed to pollutants. Higher organisms, like mammals or invertebrates, can access pollutants through the ingestion of soil material, then residual fractions might be released in the gut due to chemical conditions (Umeh *et al.,* 2017). Thus, it is essential to keep in mind that the ISO/TS 16751 norm is a tool that allows the estimation of the environmental availability in general, but does not represent bioavailability to all types of organisms. In the topic of soils/sediments remediation, bioavailability is now assimilated to the availability to microorganisms such as bacteria and fungi, and it is based on the assumption that the rapidly desorbable fraction (i.e. the bioavailable fraction) of a contaminant represents the endpoint of bioremediation (Hu *et al.,* 2014). This precision is important because as was demonstrated by Hu *et al.,* (2014), a pollution's removal through bioremediation can sometimes be higher than could have been predicted through bioavailability measurement. Thus it is important to keep in mind that bioavailability is a tool that should be used as a complement to other decision-making tools.

Nevertheless, now that the norm exists, the scientific community should start implementing it in soils/sediments remediation studies.

On one hand, because when countries are equipped with a legislation regulating environmental pollution and setting remediation goals, endpoints are established on the assumption that when environmental harm has been done, it has to be repaired to the furthest possible extent. Even for some countries where legislation is based on risk-assessment (e. g. Canada, New Zealand, Australia, USA, UK, the Netherlands, or Belgium), the total extractable content is at the basis of management. For example, in Belgium (Walloon region), the management strategy of a brownfield is based on a risk-analysis. Coefficients based on exposition scenarios, toxicological data, soil's physico-chemical properties… are applied to a content to which the targets (e.g. humans) are considered to be exposed, leading to a value that is then considered acceptable or not. The content to which coefficients are applied is assumed to be a pollutant's total extractable concentration. But this assumption has been thought to overestimate risks for some time now. As discussed previously, the interactions between a pollutant and the matrix it is in are complex and tend to become stronger with time. This means that the complete removal of a pollution can become technologically infeasible or very expensive as time goes by. It also means that risks could be overestimated if the risk-analysis estimates that the total extractable pollutant content is bioavailable to organisms (which is potentially true in case of ingestion, but not in case of dermal contact, e.g.). So complete removal of a pollution could actually be unnecessary, in some cases.

Several authors have discussed this issue, and suggested several information should be used in the decision-making: the pollutants total concentrations (based on classical exhaustive extraction methods) of course, but also their bioavailability. Norm ISO/TS 16751 is very useful to determine the environmental availability, but it should be complemented with biological assays, or chemical surrogates suitable for different biological groups, such as mammals (Alexander, 2000; Latawiec *et al.,* 2011; Duan *et al.,* 2015). And though progress still needs to be done to develop such methods, it is encouraging to know that some work has already been accomplished on PAHs bioavailability in food using in vitro digestion, and that it could be implemented on soils/sediments too (Hamidi *et al.,* 2016).

On the other hand, implementing bioavailability measurement in soils/sediments remediation studies would bring considerable insight to the processes taking place during trials. The bioavailability concept has originally been studied to explain the lack of proper biodegradation during bioremediation. Lately, the assessment of bioavailability has largely been discussed as a tool for risk-analysis in contaminated land management, as explained previously. But it should also be used as a tool to follow the evolution of that risk throughout the actual remediation process, and not only as a way to plan the extent of clean-up that should be achieved. This would mean using bioavailability assessment for all types of remediation techniques, on all types of soils/sediments being remediated. Throughout remediation research, many trials and methods have based their strategy on increasing bioavailability. State-of-the-art reviews on techniques enhancing bioavailability exist (Ortega-Calvo *et al.,* 2013) or are included into remediation reviews which evaluate progress in PAHs remediation treatments (Gan *et al.,* 2009; Kuppusamy *et al.,* 2017; Lamichhane *et al.,* 2017; Sarma *et al.,* 2019). But none of these reviews, to the best of our knowledge, have reported systematic assessment of bioavailability throughout remediation trials. As exposed throughout this section, bioavailability is at the centre or risk-analysis because it is what makes a pollutant a danger to its environment or not. So, the determination, but also the evolution of a pollutant's bioavailability should be taken into account in remediation studies. From a remediation point-of-view, it would bring considerable insight to the processes at work and help understand the dynamics of the treatment, and from a risk-analysis point-of-view, it would bring continuous data to feed the risk-analysis assessments, and could be used in combination with the total concentration contents to follow the evolution of land clean-up, the evolution of risk, and determine where to stop. Evidently, since bioavailability applies by essence to historical pollution to evaluate its danger, such work has to be associated to aged material, as it realistically presents pollutants with lowered bioavailability. Lately, a few studies have started to assess the bioavailability of PAHs in soils/sediments after undergoing remediation. Posada-Baquero *et al.,* (2019, 2020) recently applied the ISO/TS 16751 norm to determine the environmental availability of PAHs in aged-contaminated soils before and after remediation treatments, and our team measured PAHs bioaccessibility throughout a bioremediation trial (Davin *et al.,* 2019) and a rhizoremediation trial (Davin *et al.,* 2020) before the norm came out.

Finally, let us keep in mind that if, as suggested previously, intermediate PAHs metabolites or other PACs were to be added to the list of compounds of interest in the matter of environmental remediation, their bioavailability would also have to be monitored throughout remediation processes. Indeed, it was previously mentioned that some of these compounds being more polar, they are also probably more mobile and bioavailable. This would have to be verified, as it is crucial that remediation techniques actually diminish the general toxicity and threat pollutions pose towards the environment. Here again, some searchers (Hu *et al.,* 2014) have already started to examine this issue when they investigated possible links between the biodegradable and the desorbable fractions of compounds such as oxy-PAHs, but also apolar PAHs' degradation metabolites. They obtained mixed results depending on the type of compound and concluded that though bioremediation could generate genotoxic metabolites, those compounds were not necessarily desorbable from the soil, and thus bioavailable. Such statement is yet another argument to the need to implement and enlarge the assessment of bioavailability in remediation trials.

7. Conclusions

This paper has reviewed and questioned a few scientific parameter choices that have been leading soils and sediments PAHs remediation studies and management for the past decades.

The first parameter is the list of PAHs compounds that are being studied and targeted in scientific literature. We have shown that the classical 16 US-EPA compounds might no longer be sufficient to meet actual environmental challenges and quality guidelines throughout the world. We suggest that it might be relevant to enlarge the variety of studied and remediated PAHs, but also PACs, in soils/sediments to meet remediation challenges and prevent toxic dead-ends.

The second parameter is the choice of experimental material in remediation studies. We have shown with bibliometric measures that neither co-contaminated nor aged-contaminated material are systematically used in PAHs remediation trials yet, even though such material is the most representative of realistic remediation challenges when it comes to land management. We thus suggest that searchers start using aged-contaminated and co-contaminated material more systematically in their trials. We also strongly advise that all types of results, even inconclusive ones, be shared with the scientific community.

The final parameter concerns the use of bioavailability measurement. A norm was just published that allows the evaluation of environmental availability (ISO/TS 16751). It has mainly been developed as a tool to improve risk-analysis based management of contaminated land, but we suggest such measurement should be systematically included in remediation trials, on realistic soil material, to improve the comprehension of remediation processes as well as management tools.

8. References

Alexander, M., 2000. Aging, bioavailability, and overestimation of risk from environmental pollutants. *Environ. Sci. Technol.*, 34(20), 4259–4265. https://doi.org/10.1021/es001069+

Andersson, J., Achten, C., 2015. Time to say goodbye to the 16 EPA PAHs? Toward an up-to-fate use of PACs for environmental purposes. *Polycycl Aromat Comp.,* 35, 330-354.

Bleeker, E. A. J., Van Der Geest, H. G., Klamer, H. J. C., De Voogt, P., Wind, E., & Kraak, M. H. S., 1999. Toxic and Genotoxic Effects of Azaarenes : Isomers and Metabolites. *Polycycl Aromat Comp.,* 13(3), 191–203. https://doi.org/10.1080/10406639908020563

Bouchez, M., Blanchet, D., & Vandecasteele, J. P., 1995. Degradation of polycyclic aromatic hydrocarbons by pure strains and by defines strain associations: inhibition phenomena and cometabolism. *Appl. Microbiol. Biotechnol.,* 43, 156–164. https://doi.org/10.1007/BF00170638

Bowman, D. T., Jobst, K. J., Helm, P. A., Kleywegt, S., Diamond, M. L., 2019. Characterization of Polycyclic Aromatic Compounds in Commercial Pavement Sealcoat Products for Enhanced Source Apportionment. *Environ. Sci. Technol.,* 53(6), 3157–3165. https://doi.org/10.1021/acs.est.8b06779

Cachada, A., Pereira, R., da Silva, E. F., Duarte, A. C., 2014. The prediction of PAHs bioavailability in soils using chemical methods: State of the art and future challenges. *Sci. Total Environ.,* 472, 463-480. https://doi.org/10.1016/j.scitotenv.2013.11.038

CCME, 2020. Canadian Council of Ministers of the Environment, Canadian Environmental Quality Guidelines, consulted March 20th, 2020. http://ceqg-rcqe.ccme.ca/fr/index.html#void

Chibwe, L., Geier, M. C., Nakamura, J., Tanguay, R. L., Aitken, M. D., & Massey Simonich, S. L. M., 2015. Aerobic bioremediation of PAH contaminated soil results in increased genotoxicity and developmental toxicity. *Environ. Sci. Technol.,* 49, 13889−13898 https://doi.org/10.1021/acs.est.5b00499

Chibwe, L., Davie-Martin, C. L., Aitken, M. D., Hoh, E., & Massey Simonich, S. L., 2017. Identification of polar transformation products and high molecular weight polycyclic aromatic hydrocarbons (PAHs) in contaminated soil following bioremediation. *Sci. Total Environ.,* 599–600, 1099–1107. https://doi.org/10.1016/j.scitotenv.2017.04.190

Cochran, R. E., Dongari, N., Jeong, H., Beránek, J., Haddadi, S., Shipp, J., Kubátová, A., 2012. Determination of polycyclic aromatic hydrocarbons and their oxy-, nitro-, and hydroxy-oxidation products. *Anal. Chim. Acta,* 740, 93–103. https://doi.org/10.1016/j.aca.2012.05.050

Cui, X., Mayer, P., & Gan, J., 2013. Methods to assess bioavailability of hydrophobic organic contaminants: Principles, operations, and limitations. *Environ. Pollut.*, 172; 223-234. https://doi.org/10.1016/j.envpol.2012.09.013

Davie-Martin, C.L., Stratton, K.G., Teeguarden, J.G., Waters, K.M., Simonich, S.L. M., 2017. Implications of Bioremediation of Polycyclic Aromatic Hydrocarbon- Contaminated Soils for Human Health and Cancer Risk. *Environ. Sci. Technol.*, 51(17), 9458–9468. https://doi.org/10.1021/acs.est.7b02956

Davin, M., Starren, A., Marit, E., Lefébure, K., Fauconnier, M-L., Colinet, G., 2019. Investigating the Effect of *Medicago sativa* L. and *Trifolium pratense* L. Root Exudates on PAHs Bioremediation in an Aged-Contaminated Soil. *Water Air Soil Pollut.*, 230-296. https://doi.org/10.1007/s11270-019-4341-4

Davin, M., Renard, E., Lefébure, K., Fauconnier, M. L., Colinet, G., 2020. Temporal evolution of PAHs bioaccessibility in an aged-contaminated soil during the growth of two *Fabaceae*. *Int. J. Environ. Res. Public Health,* 17, 4016. https://doi.org/10.3390/ijerph17114016

Deary, M. E., Ekumankama, C. C., & Cummings, S. P., 2018. Effect of lead, cadmium, and mercury co-contaminants on biodegradation in PAH-polluted soils. *L. Degrad. Dev.*, 29(6), 1583–1594. https://doi.org/10.1002/ldr.2958

Dhar, K., Subashchandrabose, S.R., Venkateswarlu, K., Krishnan, K., Megharaj, M., 2019. Anaerobic Microbial Degradation of Polycyclic Aromatic Hydrocarbons: A Comprehensive Review. *In*: de Voogt P (ed.) *Reviews of Environmental Contamination and Toxicology*. Springer Nature, Switzerland. https://doi.org/10.1007/398_2019_29

Duan, L., Naidu, R., Thavamani, P., Mealim, J., Megharaj, M., 2015. Managing long-term polycyclic aromatic hydrocarbon contaminated soils : a risk-based approach. *Environ. Sci. Pollut. Res.*, 22, 8927–8941. https://doi.org/10.1007/s11356-013-2270-0

Duffus, J. H., 2002. “Heavy metals” - A meaningless term? (IUPAC technical report). *Pure Appl. Chem.*, 74(5), 793–807. https://doi.org/10.1351/pac200274050793

EC, 2020. European Commission, Environment, Water, Water Framework Directive, consulted March 19th, 2020. https://ec.europa.eu/environment/water/water-framework/priority_substances.htm

ECHA, 2020. European Chemicals Agency, consulted March 17th, 2020. https://echa.europa.eu/legislation

EFSA, 2020. European Food Safety Authority, consulted March 19th, 2020. EC 1881/2006. COMMISSION REGULATION (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. http://www.efsa.europa.eu/en/topics/topic/chemical-contaminants

Ehlers, L. J., & Luthy, R. G., 2003. Contaminant bioavailability in improving risk assessment and remediation rests on better understanding bioavailability. *Environ. Sci. Technol.*, 37, 295–302. https://doi.org/10.1021/es032524f

EPA, 2020. United States Environmental Protection Agency, consulted March 17th, 2020. https://www.epa.gov/eg/toxic-and-priority-pollutants-under-clean-water-act

Gan, S., Lau, E. V., & Ng, H. K., 2009. Remediation of soils contaminated with polycyclic aromatic hydrocarbons (PAHs). *J. Hazard. Mater.*, 172(2–3), 532–549. https://doi.org/10.1016/j.jhazmat.2009.07.118

García-Alonso, S., Barrado-Olmedo, A. I., & Pérez-Pastor, R. M., 2012. An Analytical Method to Determine Selected Nitro-PAHs in Soil Samples by HPLC With Fluorescence Detection. *Polycycl Aromat Comp.*, 32(5), 669–682. https://doi.org/10.1080/10406638.2012.725196

Ghosal, D., Ghosh, S., Dutta, T. K., & Ahn, Y. (2016). Current state of knowledge in microbial degradation of polycyclic aromatic hydrocarbons (PAHs): A review. *Front Microbiol.,*, 7(1369). https://doi.org/10.3389/fmicb.2016.01369

Hamidi, E. N., Hajeb, P., Selamat, J., & Razis, A. F. A., 2016. Polycyclic aromatic hydrocarbons (PAHs) and their bioaccessibility in meat: A tool for assessing human cancer risk. *Asian Pac J Cancer P.*, 17(1), 15–23. https://doi.org/10.7314/APJCP.2016.17.1.15

Hu, J., Nakamura, J., Richardson, S. D., & Aitken, M. D., 2012. Evaluating the effects of bioremediation on genotoxicity of polycyclic aromatic hydrocarbon-contaminated soil using genetically engineered, higher eukaryotic cell lines. *Environ. Sci. Technol.*, 46, 4607-4613. https://doi.org/10.1021/es300020e

Hu, J., Adrion, A. C., Nakamura, J., Shea, D., & Aitken, M. D., 2014. Bioavailability of (geno)toxic contaminants in polycyclic aromatic hydrocarbon-contaminated soil before and after biological treatment. *Environ. Eng. Sci.*, 31(4), 176–182. https://doi.org/10.1089/ees.2013.0409

Idowu, O., Semple, K. T., Ramadass, K., O’Connor, W., Hansbro, P., & Thavamani, P., 2019. Beyond the obvious: Environmental health implications of polar polycyclic aromatic hydrocarbons. *Environ. Int.*, 123, 543–557. https://doi.org/10.1016/j.envint.2018.12.051

Iqbal, J., Overton, E. B., & Gisclair, D., 2008. Polycyclic aromatic hydrocarbons in Louisiana rivers and coastal environments: Source fingerprinting and forensic analysis. *Environ. Forensics*, 9(1), 63–74. https://doi.org/10.1080/15275920801888301

ISO/TS 16751:2018. Soil quality - Environmental availability of non-polar organic compounds - Determination of the potential bioavailable fraction and the non-bioavailable fraction using a strong adsorbent or complexing agent.

ISO 17402:2008. Soil quality - Requirements and guidance for the selection and application of methods for the assessment of bioavailability of contaminants in soil and soil materials.

Keith, L.H., 2015. The Source of U.S. EPA's Sixteen PAH Priority Pollutants. *Polycycl Aromat Comp.,* 35:147–160. https://doi.org/10.1080/10406638.2014.892886

Kuppusamy, S., Thavamani, P., Venkateswarlu, K., Lee, Y. B., Naidu, R., Megharaj, M., 2017. Remediation approaches for polycyclic aromatic hydrocarbons (PAHs) contaminated soils : Technological constraints , emerging trends and future directions. *Chemosphere*, 168, 944–968. http://dx.doi.org/10.1016/j.chemosphere.2016.10.115

Lamichhane, S., Krishna, K. C. B., & Sarukkalige, R., 2017. Surfactant-enhanced remediation of polycyclic aromatic hydrocarbons: A review. *J. Environ. Manage.*, 199, 46-61. https://doi.org/10.1016/j.jenvman.2017.05.037

Latawiec, A. E., Swindell, A. L., Simmons, P., Reid, B. J., 2011. Bringing bioavailability into contaminated land decision making: The way forward? *Crit. Rev. Environ. Sci. Technol.*, 41(1), 52–77. https://doi.org/10.1080/00102200802641780

Lundstedt, S.,Haglund, P., Öberg, L., 2002. Degradation and formation of polycyclic aromatic compounds during bioslurry treatment of an aged gasworks soil. *Environ. Toxicol. Chem.,* 22(7), 1413-1420.

Lundstedt, S., Bandowe, B. A. M., Wilcke, W., Boll, E., Christensen, J. H., Vila, J., Grifoll, M., Faure, P., Biache, C., Lorgeoux, C., Larsson, M., Frech Irgum, K., Ivarsson, P., Ricci, M., 2014. First intercomparison study on the analysis of oxygenated polycyclic aromatic hydrocarbons (oxy-PAHs) and nitrogen heterocyclic polycyclic aromatic compounds (N-PACs) in contaminated soil. *TrAC - Trends Anal. Chem.,* 57, 83–92. https://doi.org/10.1016/j.trac.2014.01.007

Mahanty, B., Pakshirajan, K., & Dasu, V. V., 2011. Understanding the complexity and strategic evolution in PAH remediation research. *Crit. Rev. Environ. Sci. Technol.,* 41(19), 1697–1746. https://doi.org/10.1080/10643389.2010.481586

Mueller, A., Ulrich, N., Hollmann, J., Zapata Sanchez, C. E., Rolle-Kampczyk, U. E., von Bergen, M., 2019. Characterization of a multianalyte GC-MS/MS procedure for detecting and quantifying polycyclic aromatic hydrocarbons (PAHs) and PAH derivatives from air particulate matter for an improved risk assessment. *Environ. Pollut.*, 255(112967). https://doi.org/10.1016/j.envpol.2019.112967

Niederer, M., 1998. Determination of polycyclic aromatic hydrocarbons and substitutes (nitro-, oxy-PAHs) in urban soil and airborne particulate by GC-MS and NCI-MS/MS. *Environ. Sci. Pollut. Res.*, 5(4), 209–216. https://doi.org/10.1007/BF02986403

Nzila, A., 2018. Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons under anaerobic conditions: Overview of studies, proposed pathways and future perspectives. *Environ. Pollut.*, 239, 788–802. https://doi.org/10.1016/j.envpol.2018.04.074

Ortega-Calvo, J. J., Tejeda-Agredano, M. C., Jimenez-Sanchez, C., Congiu, E., Sungthong, R., Niqui-Arroyo, J. L., Cantos, M., 2013. Is it possible to increase bioavailability but not environmental risk of PAHs in bioremediation? *J. Hazard. Mater.*, 261, 733–745. https://doi.org/10.1016/j.jhazmat.2013.03.042

Ortega-Calvo, J. J., Harmsen, J., Parsons, J. R., Semple, K. T., Aitken, M. D., Ajao, C., Eadsforth, C., Galay-Burgos, M., Naidu, R., Oliver, R., Peijnenburg, W. J. G. M., Römbke, J., Streck, G., Versonnen, B., 2015. From Bioavailability Science to Regulation of Organic Chemicals. *Environ. Sci. Technol.*, 49(17), 10255–10264. https://doi.org/10.1021/acs.est.5b02412

Park, J., Ball, L. M., Richardson, S. D., Zhu, H. O., & Aitken, M. D., 2008. Oxidate mutagenicity of polar fractions from polycyclic aromatic hydrocarbon – contaminated soils *Environ. Toxicol. Chem.*, 27(11), 2207–2215.

Pazos, M., Rosales, E., Alcántara, T., Gómez, J., Sanromán, M. A., 2010. Decontamination of soils containing PAHs by electroremediation: A review. *J. Hazard. Mater.*, 177, 1-11. https://doi.org/10.1016/j.jhazmat.2009.11.055

Posada-Baquero, R., Martín, M. L., & Ortega-Calvo, J. J., 2019. Implementing standardized desorption extraction into bioavailability-oriented bioremediation of PAH-polluted soils. *Sci. Total Environ.*, 696(134011). https://doi.org/10.1016/j.scitotenv.2019.134011

Posada-Baquero, R., Jiménez-Volkerink, S. N., García, J. L., Vila, J., Cantos, M., Grifoll, M., & Ortega-Calvo, J. J., 2020. Rhizosphere-enhanced biosurfactant action on slowly desorbing PAHs in contaminated soil. *Sci. Total Environ.*, 720(137608). https://doi.org/10.1016/j.scitotenv.2020.137608

Reichenberg, F., & Mayer, P., 2006. Two complementary sides of bioavailability: accesibility and chemical activity of organic contaminants in sediments and soils. *Environ. Toxicol. Chem.*, 25(5), 1239-1245. https://doi.org/10.1897/05-458R.1

Reichert, G., Hilgert, S., Fuchs, S., Azevedo, J. C. R., 2019. Emerging contaminants and antibiotic resistance in the different environmental matrices of Latin America. *Environ. Pollut.*, 255(113140). https://doi.org/10.1016/j.envpol.2019.113140

Ren, X., Zeng, G., Tang, L., Wang, J., Wan, J., Liu, Y., Yu, J., Yi, H., Ye, S., Deng, R., 2018. Sorption, transport and biodegradation – An insight into bioavailability of persistent organic pollutants in soil. *Sci. Total Environ.*, 610-611, 1154–1163. http://dx.doi.org/10.1016/j.scitotenv.2017.08.089

Riding, M. J., Doick, K. J., Martin, F. L., Jones, K. C., Semple, K. T., 2013. Chemical measures of bioavailability/bioaccessibility of PAHs in soil: Fundamentals to application. *J. Hazard. Mater.*, 261, 687–700. https://doi.org/10.1016/j.jhazmat.2013.03.033

Sanseverino, J., Werner, C., Fleming, J., Applegate, B., King, J. M. H., Sayler, G. S., 1993. Molecular diagnostics of polycyclic aromatic hydrocarbon biodegradation in manufactured gas plant soils. *Biodegradation*, 4, 303–321.

Sarma, H., Nava, A. R., & Prasad, M. N. V., 2019. Mechanistic understanding and future prospect of microbe-enhanced phytoremediation of polycyclic aromatic hydrocarbons in soil. *Environ. Technol. Innov.*, 13, 318–330. https://doi.org/10.1016/j.eti.2018.12.004

Semple, K. T., Morriss, A. W. J., & Paton, G. I., 2003. Bioavailability of hydrophobic organic contaminants in soils: fundamental concepts and techniques for analysis. *Eur. J. Soil Sci.*, 54, 809–818. https://doi.org/10.1046/j.1365-2389.2003.00564.x

Semple, K. T., Doick, K. J., Jones, K. C., Burauel, P., Craven, A., Harms, H., 2004. Peer Reviewed: Defining Bioavailability and Bioaccessibility of Contaminated Soil and Sediment is Complicated. *Environ. Sci. Technol.*, 38(12), 228A-231A. https://doi.org/10.1021/es040548w

Semple, K. T., Doick, K. J., Wick, L. Y., Harms, H., 2007. Microbial interactions with organic contaminants in soil: Definitions, processes and measurement. *Environ. Pollut.*, 150(1), 166–176. https://doi.org/10.1016/j.envpol.2007.07.023

Stringfellow, W. T., & Aitken, M. D., 1995. Competitive metabolism of naphthalene, methylnaphthalenes, and fluorene by phenanthrene-degrading pseudomonads. *Appl. Environ. Microbiol.*, 61(1), 357–362. https://doi.org/10.1128/aem.61.1.357-362.1995

Tian, Z.; Gold, A.; Nakamura, J.; Zhang, Z.; Vila, J.; Singleton, D.R.; Collins, L.; Aitken, M.D., 2017a. Non-target analysis reveals a bacterial metabolite of pyrene implicated in the genotoxicity of contaminated soil after bioremediation. *Environ. Sci. Technol.*, 51(12), 7091-7100. https://doi.org/10.1021/acs.est.7b01172

Tian, Z.; Vila, J.; Wang, H.; Bodnar, W.; Aitken, M.D., 2017b. Diversity and abundance of high-molecular-weight azaarenes in PAH-contaminated environmental samples. *Environ. Sci. Technol.*, 51(24), 14047−14054. https://doi.org/10.1021/acs.est.7b03319

Umeh, A. C., Duan, L., Naidu, R., Semple, K. T., 2017. Residual hydrophobic organic contaminants in soil: Are they a barrier to risk-based approaches for managing contaminated land? *Environ. Int.*, 98, 18–34. https://doi.org/10.1016/j.envint.2016.09.025

Vila, J., Tauler, M., & Grifoll, M., 2015. Direct bacterial PAH degradation in marine and terrestrial habitats. *Curr. Opin. Biotechnol.*, 33, 95–102. http://dx.doi.org/10.1016/j.copbio.2015.01.006

Von Lau, E., Gan, S., Ng, H. K., Poh, P. E., 2014. Extraction agents for the removal of polycyclic aromatic hydrocarbons (PAHs) from soil in soil washing technologies. *Environ. Pollut.*, 184, 640-649. https://doi.org/10.1016/j.envpol.2013.09.010

Wang, G., Jiang, Y., Hu, S., Guo, Y., 2018. Aspartic acid-β-cyclodextrin-assisted phytoremediation of soil cocontaminated with cadmium and fluorene using alfalfa (*Medicago sativa* L.). *Environ. Eng. Sci.*, 35(4), 279–288. https://doi.org/10.1089/ees.2016.0460

WHO, 2020. World Health Organization, International Program on Chemical Safety, Environmental Health Criteria, consulted March 20th, 2020. http://www.inchem.org/documents/ehc/ehc/ehc202.html

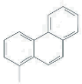
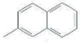
Wickrama-Arachchige, A. U. K., Hirabayashi, T., Imai, Y., Guruge, K. S., Dharmaratne, T. S., Ohura, T., 2020. Accumulation of halogenated polycyclic aromatic hydrocarbons by different tuna species, determined by high-resolution gas chromatography Orbitrap mass spectrometry. *Environ. Pollut.*, 256(113487). https://doi.org/10.1016/j.envpol.2019.113487

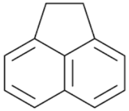
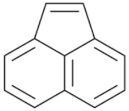
Yu, L., Duan, L., Naidu, R., Semple, K. T., 2018. Abiotic factors controlling bioavailability and bioaccessibility of polycyclic aromatic hydrocarbons in soil: Putting together a bigger picture. *Sci. Total Environ.*, 613–614, 1140–1153. https://doi.org/10.1016/j.scitotenv.2017.09.025

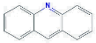
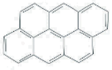
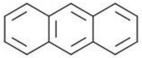
9. Supplementary material

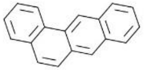
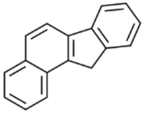
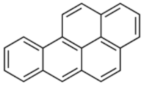
9.1. Comparison of PAHs watch lists

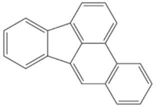
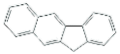
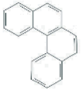
Supplementary table 5. Comparison of the polycyclic aromatic compounds (polar and apolar) mentioned in several watch lists throughout the world.

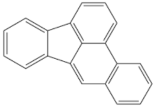
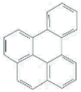
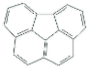
Compound	Structure	Watch list						
		WHO[1]	US-EPA[2]	CCME[3]	European Union			
					Food Regulation[4]	WFD[5]	REACH Regulation[6]	POPs Regulation[6]
1-methylphenanthrene		x						
2-methylnaphthalene				x				

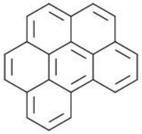
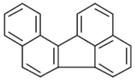
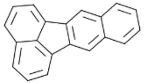
Compound	Structure	Watch list						
		WHO[1]	US-EPA[2]	CCME[3]	European Union			
					Food Regulation[4]	WFD[5]	REACH Regulation[6]	POPs Regulation[6]
5-methylchrysene		x			x			
Acenaphthene		x	x	x				
Acenaphtylene		x	x	x				

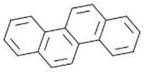
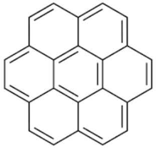
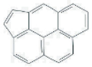
Compound	**Structure**	**Watch list**						
		WHO[1]	**US-EPA**[2]	**CCME**[3]	**European Union**			
					Food Regulation[4]	**WFD**[5]	**REACH Regulation**[6]	**POPs Regulation**[6]
Acridine				x				
Anthanthrene		x						
Anthracene		x	x	x		x	x	

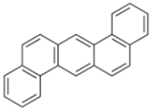
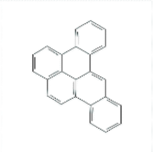
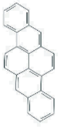
Compound	**Structure**	**Watch list**						
		WHO[1]	**US-EPA[2]**	**CCME[3]**	**European Union**			
					Food Regulation[4]	**WFD[5]**	**REACH Regulation[6]**	**POPs Regulation[6]**
Benzo[a]anthracene		x	x	x	x		x	
Benzo[a]fluorene		x						
Benzo[a]pyrene		x	x	x	x	x	x	x

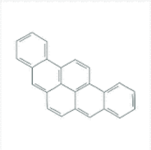
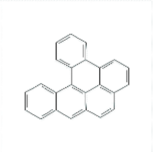
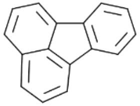
Compound	**Structure**	**Watch list**						
		WHO[1]	**US-EPA**[2]	**CCME**[3]	**European Union**			
					Food Regulation[4]	**WFD**[5]	**REACH Regulation**[6]	**POPs Regulation**[6]
Benzo[b] fluoranthene		x	x	x	x	x		
Benzo[b]fluorene		x						
Benzo[c] phenanthrene		x						

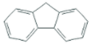
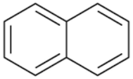
Compound	Structure	Watch list						
		WHO[1]	US-EPA[2]	CCME[3]	European Union			
					Food Regulation[4]	WFD[5]	REACH Regulation[6]	POPs Regulation[6]
Benzo[e] acephenanthrylene								x
Benzo[e]pyrene		x						
Benzo[ghi] fluoranthene		x						

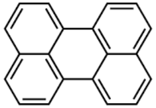
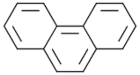
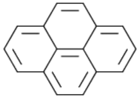
Compound	**Structure**	**Watch list**						
		WHO[1]	**US-EPA**[2]	**CCME**[3]	**European Union**			
					Food Regulation[4]	**WFD**[5]	**REACH Regulation**[6]	**POPs Regulation**[6]
Benzo[ghi]perylene		x	x		x	x	x	
Benzo[j] fluoranthene		x			x			
Benzo[k] fluoranthene		x	x	x	x	x	x	x

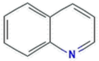
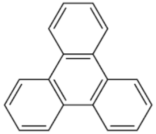
Compound	**Structure**	**Watch list**						
		WHO[1]	**US-EPA**[2]	**CCME**[3]	**European Union**			
					Food Regulation[4]	**WFD**[5]	**REACH Regulation**[6]	**POPs Regulation**[6]
Chrysene		x	x	x	x		x	
Coronene		x						
Cyclopenta[c,d] pyrene		x			x			

Compound	**Structure**	**Watch list**						
		WHO[1]	**US-EPA**[2]	**CCME**[3]	**European Union**			
					Food Regulation[4]	**WFD**[5]	**REACH Regulation**[6]	**POPs Regulation**[6]
Dibenz[a,h] anthracene		x	x	x	x			
Dibenzo[a,e]pyrene		x			x			
Dibenzo[a,h]pyrene		x			x			

Compound	**Structure**	**Watch list**						
		WHO[1]	**US-EPA**[2]	**CCME**[3]	**European Union**			
					Food Regulation[4]	**WFD**[5]	**REACH Regulation**[6]	**POPs Regulation**[6]
Dibenzo[a,i]pyrene		x			x			
Dibenzo[a,l]pyrene		x			x			
Fluoranthene		x	x	x		x	x	

Compound	**Structure**	**Watch list**						
		WHO[1]	**US-EPA**[2]	**CCME**[3]	**European Union**			
					Food Regulation[4]	**WFD**[5]	**REACH Regulation**[6]	**POPs Regulation**[6]
Fluorene		x	x	x				
Indeno [1,2,3-cd]pyrene		x	x	x	x	x		x
Naphthalene		x	x	x		x		

Compound	Structure	Watch list						
		WHO[1]	US-EPA[2]	CCME[3]	European Union			
					Food Regulation[4]	WFD[5]	REACH Regulation[6]	POPs Regulation[6]
Perylene		x						
Phenanthrene		x	x	x			x	
Pyrene		x	x	x			x	

Compound	**Structure**	**Watch list**						
		WHO[1]	**US-EPA**[2]	**CCME**[3]	**European Union**			
					Food Regulation[4]	**WFD**[5]	**REACH Regulation**[6]	**POPs Regulation**[6]
Quinoline				x				
Triphenylene		x						

Sources
[1]WHO, 2020; [2]EPA, 2020; [3]CCME, 2020; [4]EFSA, 2020; [5]EC, 2020; [6]ECHA, 2020.

9.2. Bibliometric research

The databases exposed in Supplementary table 6 and Supplementary table 7 were searched for scientific publications between February 17th and 21st 2020. There was no restriction on the type of publication (book, article, report…) but the results were limited to English, until December 31st 2019. Besides, the search terms were limited, when possible, to the title, abstract, and keywords.

Most databases accept the truncation (*) for search terms. However, when they did not, the general results that appeared from a truncation use in a database (e.g. Scopus) had to be developed and are described after the "=" symbol.

The utilized search items are listed hereunder:

- pah* or polycyclic aromatic hydrocarbon*
- soil* or sediment*
- *remediation = remediation or bioremediation or phytoremediation
- multicontam* or polycontam* or cocontam*
- aged or historical or former or ancient
- natural attenuation
- heating
- electrokinetic or electrochemical
- washing
- solubilisation
- chemical oxidation
- photocatalytic degradation
- bioremediation
- phytoremediation
- bioavailability or availability or phytoavailability or accessibility or bioaccessibility

Several research questions, listed hereunder, were run through each database:

1. (pah* or polycyclic aromatic hydrocarbon*)
2. (pah* or polycyclic aromatic hydrocarbon*)

   and (soil* or sediment*)
3. (pah* or polycyclic aromatic hydrocarbon*)

   and (soil* or sediment*)

   and (*remediation = remediation or bioremediation or phytoremediation)
4. (pah* or polycyclic aromatic hydrocarbon*)

   and (soil* or sediment*)

   and (*remediation = remediation or bioremediation or phytoremediation)

   and (multicontam* or polycontam* or cocontam*)
5. (pah* or polycyclic aromatic hydrocarbon*)

   and (soil* or sediment*)

   and (*remediation = remediation or bioremediation or phytoremediation)

   and (multicontam* or polycontam* or cocontam*)

   and (aged or historical or former or ancient)
6. (pah* or polycyclic aromatic hydrocarbon*)

   and (soil* or sediment*)

   and (*remediation = remediation or bioremediation or phytoremediation)

   and (aged or historical or former or ancient)

7. (pah* or polycyclic aromatic hydrocarbon*)

   and (soil* or sediment*)

   and (*remediation = remediation or bioremediation or phytoremediation)

   and (heating)

8. (pah* or polycyclic aromatic hydrocarbon*)

   and (soil* or sediment*)

   and (*remediation = remediation or bioremediation or phytoremediation)

   and (heating)

   and (aged or historical or former or ancient)

9. (pah* or polycyclic aromatic hydrocarbon*)

   and (soil* or sediment*)

   and (*remediation = remediation or bioremediation or phytoremediation)

   and (electrokinetic or electrochemical)

10. (pah* or polycyclic aromatic hydrocarbon*)

    and (soil* or sediment*)

    and (*remediation = remediation or bioremediation or phytoremediation)

    and (electrokinetic or electrochemical)

    and (aged or historical or former or ancient)

11. (pah* or polycyclic aromatic hydrocarbon*)

    and (soil* or sediment*)

    and (*remediation = remediation or bioremediation or phytoremediation)

    and (washing)

12. (pah* or polycyclic aromatic hydrocarbon*)
    and (soil* or sediment*)
    and (*remediation = remediation or bioremediation or phytoremediation)
    and (washing)
    and (aged or historical or former or ancient)
13. (pah* or polycyclic aromatic hydrocarbon*)
    and (*remediation = remediation or bioremediation or phytoremediation)
    and (soil* or sediment*)
    and (solubilization)
14. (pah* or polycyclic aromatic hydrocarbon*)
    and (soil* or sediment*)
    and (*remediation = remediation or bioremediation or phytoremediation)
    and (solubilization)
    and (aged or historical or former or ancient)
15. (pah* or polycyclic aromatic hydrocarbon*)
    and (soil* or sediment*)
    and (*remediation = remediation or bioremediation or phytoremediation)
    and (chemical oxidation)

16. (pah* or polycyclic aromatic hydrocarbon*)

    and (soil* or sediment*)

    and (*remediation = remediation or bioremediation or phytoremediation)

    and (chemical oxidation)

    and (aged or historical or former or ancient)

17. (pah* or polycyclic aromatic hydrocarbon*)

    and (soil* or sediment*)

    and (bioremediation)

18. (pah* or polycyclic aromatic hydrocarbon*)

    and (soil* or sediment*)

    and (bioremediation)

    and (aged or historical or former or ancient)

19. (pah* or polycyclic aromatic hydrocarbon*)

    and (soil* or sediment*)

    and (phytoremediation)

20. (pah* or polycyclic aromatic hydrocarbon*)

    and (soil* or sediment*)

    and (phytoremediation)

    and (aged or historical or former or ancient)

Several proportions of publication numbers were calculated, as exposed in Supplementary table 6 and Supplementary table 7. In both tables, the question numbers represent searching questions previously exposed, and the calculated proportions (expressed as a fraction between two question numbers), represent the proportion of publications concerning a specific research question compared to another one.

For example in Supplementary table 7, the number of publications concerning PAHs remediation in soils/sediments was narrowed down to a category of treatments (heating, electrokinetic or electrochemical, washing, solubilisation, chemical oxidation, bioremediation and phytoremediation), then it was narrowed again with terms related to the aged character of the pollution, respectively leading to a number A and a number B of publications. The proportion of documents focussing on aged experimental material was then calculated (B/A). The only exceptions were for the "bioremediation" and "phytoremediation" strategies for which the searching term "remediation" was removed from the searching question in the database, as it is included in the term.

Note: It is important to precise that the searching terms "aged", "former", "ancient, and "historical" were applied in database researches to discriminate the publications based on actual aged material from the publications based on artificially aged material, which is very common. Typically, an uncontaminated soil is harvested, dried and sieved, spiked with known amounts of one or several contaminants, then left "to age" in sealed boxes for a few weeks to a few months (Wang *et al.,* 2018). As mentioned earlier, ageing processes complicate greatly the remediation of pollutants. Because aged-contaminated soils/sediments have usually been in contact with the pollution for months, years, or even decades, the pollutants are highly partitioned into soil material. Factors of natural ageing are abiotic and biotic influences such as heat, moisture, oxygen, biota and microbiota… (Yu *et al.,* 2018). The combination of all these factors and their variation is what makes ageing a long and slow process, variable both in speed and extent, and thus brings a lot of complexity and diversity between aged-contaminated soils/sediments. So, to our sense, the best way to develop remediation treatments suitable for realistic contaminated soils/sediments is to work on realistic aged samples, which there is an abundance of, unfortunately. However, no bibliographic search question nor tool is perfect, and it is possible that by applying the "aged" searching terms, some publications working on actual aged material did not appear. However, there is no way to discriminate these documents from the ones based on artificially contaminated material given the keywords that appear in their title, keywords section and abstract. We would like to apologize to these authors, as the objective of such bibliometric research is to make a statement, not discriminate quality work.

Supplementary table 6. a. Number of documents published in English on PAHs (1), on PAHs in soils or sediments (2), on the remediation of PAHs in soils or sediments (3), on the remediation of PAHs in soils or sediments presenting multiple contaminations (4), and on the remediation of PAHs in aged soils or sediments presenting multiple contaminations (5) in a series of databases until the end of year 2019.

Number of documents	Question number	1	2	3	4	5
	Searching terms	pah* or polycyclic aromatic hydrocarbon*				
			soil* or sediment*			
				*remediation = remediation + bioremediation + phytoremediation		
					multicontam* or polycontam* or cocontam*	
						aged or historical or former or ancient
Database						
AGRICOLA		17613	5735	1404	0	0
Agricu. & Environ. Science Collection		87248	28789	6267	2	0
Agricu. Science Collection		20514	6891	1661	0	0
Agriculture Science Database		2901	1156	260	0	0
ASP		47249	6150	1407	2	0
CAB ABST		29566	10886	2629	4	0

(continued)

Number of documents	**Question number**	1	2	3	4	5
	Searching terms	pah* or polycyclic aromatic hydrocarbon*	soil* or sediment*	*remediation = remediation + bioremediation + phytoremediation	multicontam* or polycontam* or cocontam*	aged or historical or former or ancient
Database						
Environment Complete		24177	8650	1992	4	0
Environmental Science Database		9781	3574	795	0	0
Environmental Science Index		66508	22466	4692	2	0
GreenFILE		11936	4648	917	1	0
Medline		20474	4963	884	2	0
Scopus		70111	13368	3283	6	0
TOXLINE		15765	4044	732	0	0

Supplementary table 6. b. Proportions of the numbers of documents published in English on several topics compared to a larger pool of documents. The numbers figuring in the calculated proportions line represent the number of a search question exposed in part a of the table.

	Calculated proportions	2/1	3/1	3/2	4/3	5/3
Proportion of documents	**Searching terms**	pah* or polycyclic aromatic hydrocarbon*				
		soil* or sediment*				
			*remediation = remediation + bioremediation + phytoremediation			
					multicontam* or polycontam* or cocontam*	
						aged or historical or former or ancient
Database						
AGRICOLA		33%	8%	24%	0,00%	0,00%
Agricu. & Environ. Science Collection		33%	7%	22%	0,03%	0,00%
Agricu. Science Collection		34%	8%	24%	0,00%	0,00%
Agriculture Science Database		40%	9%	22%	0,00%	0,00%
ASP		13%	3%	23%	0,14%	0,00%
CAB ABST		37%	9%	24%	0,15%	0,00%
Environment Complete		36%	8%	23%	0,20%	0,00%

(continued)

	Calculated proportions	2/1	3/1	3/2	4/3	5/3
Proportion of documents	**Searching terms**	pah* or polycyclic aromatic hydrocarbon*				
		soil* or sediment*				
			*remediation = remediation + bioremediation + phytoremediation			
					multicontam* or polycontam* or cocontam*	
						aged or historical or former or ancient
Database						
Environmental Science Collection		33%	7%	21%	0,04%	0,00%
Environmental Science Database		37%	8%	22%	0,00%	0,00%
Environmental Science Index		34%	7%	21%	0,04%	0,00%
(continued)						
GreenFILE		39%	8%	20%	0,11%	0,00%
Medline		24%	4%	18%	0,23%	0,00%
Scopus		19%	5%	25%	0,18%	0,00%
TOXLINE		26%	5%	18%	0,00%	0,00%
mean		**31%**	**7%**	**22%**	**0,08%**	**0,00%**
sd		8%	2%	2%	0,08%	0,00%
CI (5%)		**4%**	**1%**	**1%**	**0,04%**	**0,00%**

Supplementary table 7. a. Number of documents published in English on the remediation of PAHs in soils or sediments (3), on the remediation of PAHs in aged soils or sediments (6), and on specific treatments of PAHs in soils or sediments. Searching numbers 7, 9, 11, 13, 15, 17, and 19 are for specific treatments in soils or sediments in general, and searching numbers 8, 10, 12, 14, 16, 18, and 20 are for specific treatments in aged soils or sediments.

Number of documents	Question number	3	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
	Searching terms	pah* or polycyclic aromatic hydrocarbon*															
		soil* or sediment*															
		*remediation = remediation + bioremediation + phytoremediation												/			
				heating		electrokinetic or electrochemical		washing		solubilization		chemical oxidation		bioremediation		phytoremediation	
			aged or historical or former or ancient		aged or historical or former or ancient		aged or historical or former or ancient		aged or historical or former or ancient		aged or historical or former or ancient		aged or historical or former or ancient		aged or historical or former or ancient		aged or historical or former or ancient
Database																	
AGRICOLA		1404	158	8	1	27	2	1	0	1	0	45	8	674	75	278	31
Agricu. & Environ. Science Collection		6267	808	48	14	159	12	24	5	12	2	197	33	3640	471	1109	139
Agricu. Science Collection		1661	191	12	2	37	2	3	1	3	0	55	11	833	98	320	38
(continued) Agriculture Science Database		260	33	4	1	10	0	2	1	2	0	10	3	159	23	42	7

Number of documents																
Question number	3	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Searching terms	pah* or polycyclic aromatic hydrocarbon*															
	soil* or sediment*															
	*remediation = remediation + bioremediation + phytoremediation														/	
			heating		electrokinetic or electrochemical		washing		solubilization		chemical oxidation		bioremediation		phytoremediation	
		aged or historical or former or ancient		aged or historical or former or ancient		aged or historical or former or ancient		aged or historical or former or ancient		aged or historical or former or ancient		aged or historical or former or ancient		aged or historical or former or ancient		aged or historical or former or ancient
Database																
ASP	1407	172	19	3	58	5	48	6	55	4	34	9	779	98	244	28
CAB ABST	2629	319	18	4	71	5	75	9	61	8	49	9	1383	175	460	49
Environment Complete	1992	241	20	7	72	6	61	9	56	7	49	12	1056	128	280	34
Environmental Science Collection	4738	641	39	13	132	10	22	5	11	2	151	25	2884	390	816	107
Environmental Science Database	795	97	7	3	19	2	4	1	4	1	22	6	455	55	167	22
Environmental Science Index	4692	634	39	13	130	10	22	5	11	2	151	25	2856	386	813	106
(continued) GreenFILE	917	116	11	3	45	4	35	7	34	6	21	4	449	61	154	16
Medline	884	108	5	2	27	1	26	1	20	1	20	4	442	57	153	19
Scopus	3283	372	40	10	115	9	119	11	133	12	191	28	2194	234	484	52
TOXLINE	732	99	1	1	27	1	1	0	3	0	28	2	319	47	137	16

Supplementary table 7. b. Proportions of the numbers of documents published in English on several topics compared to a larger pool of documents. The numbers figuring in the calculated proportions line represent the number of a search question exposed in part a of the table.

Calculated proportions	6/3	7/3	8/7	9/3	10/9	11/3	12/11	13/3	14/13	15/3	16/15	17/3	18/17	19/3	20/19
Searching terms	pah* or polycyclic aromatic hydrocarbon*														
Proportion of documents	soil* or sediment*														
	*remediation = remediation + bioremediation + phytoremediation														
		heating		electrokinetic or electrochemical		washing		solubilization		chemical oxidation		bioremediation		phytoremediation	
	aged or historical or former or ancient		aged or historical or former or ancient		aged or historical or former or ancient		aged or historical or former or ancient		aged or historical or former or ancient		aged or historical or former or ancient		aged or historical or former or ancient		aged or historical or former or ancient
Database															
AGRICOLA	11%	0%	13%	0%	7%	0%	0%	0%	0%	1%	18%	12%	11%	5%	11%
Agricu. & Environ. Science Collection	13%	0%	29%	1%	8%	0%	21%	0%	17%	1%	17%	13%	13%	4%	13%
Agricu. Science Collection	11%	0%	17%	1%	5%	0%	33%	0%	0%	1%	20%	12%	12%	5%	12%
Agriculture Science Database	13%	0%	25%	1%	0%	0%	50%	0%	0%	1%	30%	14%		4%	
ASP	12%	0%	16%	1%	9%	1%	13%	1%	7%	1%	26%	13%	13%	4%	11%
CAB ABST	12%	0%	22%	1%	7%	1%	12%	1%	13%	0%	18%	13%	13%	4%	11%
Environment Complete	12%	0%	35%	1%	8%	1%	15%	1%	13%	1%	24%	12%	12%	3%	12%

	Calculated proportions	6/3	7/3	8/7	9/3	10/9	11/3	12/11	13/3	14/13	15/3	16/15	17/3	18/17	19/3	20/19
Proportion of documents	**Searching terms**	pah* or polycyclic aromatic hydrocarbon*														
		soil* or sediment*														
		*remediation = remediation + bioremediation + phytoremediation														
			heating		electrokinetic or electrochemical		washing		solubilization		chemical oxidation		bioremediation		phytoremediation	
		aged or historical or former or ancient		aged or historical or former or ancient		aged or historical or former or ancient		aged or historical or former or ancient		aged or historical or former or ancient		aged or historical or former or ancient		aged or historical or former or ancient		aged or historical or former or ancient
Database																
Environmental Science Collection		14%	0%	33%	1%	8%	0%	23%	0%	18%	1%	17%	13%	14%	4%	13%
Environmental Science Database		12%	0%	43%	1%	11%	0%	25%	0%	25%	1%	27%	13%		5%	
Environmental Science Index		14%	0%	33%	1%	8%	0%	23%	0%	18%	1%	17%	13%	14%	4%	13%
GreenFILE		13%	0%	27%	1%	9%	1%	20%	1%	18%	0%	19%	10%	14%	3%	10%
Medline		12%	0%	40%	1%	4%	1%	4%	0%	5%	0%	20%	9%	13%	3%	12%
Scopus		11%	0%	25%	1%	8%	1%	9%	1%	9%	1%	15%	16%	11%	4%	11%
TOXLINE		14%	0%	100%	1%	4%	0%	0%	0%	0%	1%	7%	8%	15%	3%	12%
mean		12%	0%	33%	1%	7%	0%	18%	0%	10%	1%	20%	12%	13%	4%	12%
sd		1%	0%	21%	0%	3%	0%	13%	0%	8%	0%	6%	2%	1%	1%	1%
CI (5%)		0,4%	0,0%	11%	0,1%	1%	0,2%	7%	0%	4%	0,1%	3%	1%	1%	0,3%	1%

General Conclusion

Discussion, conclusions, and perspectives

Two different aged-contaminated soils hosted different remediation experiments throughout this thesis. The tested treatments were either based on soil washing, soil incubation in microcosms, or rhizoremediation, and involved commercial saponin, root exudates from *M. sativa* L. or *T. pratense* L., or the whole plants. Besides, a bioaccessibility measurement protocol was adapted to each soil through the modelling of PAHs desorption kinetics. Several results and observations were gathered and discussed previously in the context of each experiment. The following discussion aims at making comparisons between the soils that were experimented on but also at making links between the results and observations that were collected. Firstly, a few comments will be made on the development of the PAHs bioaccessibility assessment protocol as well as on the initial bioaccessible PAHs contents that were measured in the experimental soils. Secondly, the discussion will focus on the impact of the different treatments on the soils' microbial health and on the PAHs' bioaccessibility and dissipation in the tested soils, as a mean to bring some answers to the working hypothesis: "Could root exudates from some plants (*M. sativa* L. or *T. pratense* L.) influence PAHs bioaccessibility, and thus enhance their bioremediation whilst being non-toxic towards the soil microbiota?" The general conclusion will be completed by a few recommendations and perspectives.

1. Assessing the PAHs bioaccessible contents

Two aged-contaminated soils were used for the experiments in this thesis. Both soils were heavily contaminated with PAHs as the initial contents of 15 PAHs were determined to range from 2.9 ± 0.1 mg kg^{-1}DW to 65.9 ± 7.1 mg kg^{-1}DW for a total of 381 ± 39.8 mg kg^{-1}DW in the soil from Saint-Ghislain (used in Part 1 and Part 2), and from 1.0 ± 0.4 mg kg^{-1}DW to 139 ± 36.6 mg kg^{-1}DW for a total of 917 ± 146 mg kg^{-1}DW in the soil from Marchienne-au-Pont (used in Part 3) (Table 15).

In order to assess the bioaccessible PAHs contents of each soil, PAHs desorption kinetics were measured and modelled according to similar protocols (Part 2 and Part 3). Both times, it is the site desorption model that was used to determine a common Tenax® extraction time (t_{ex}). All fitted parameters and calculated t_{ex} values are summarized in Table 16. When examining the values, there is no real tendency that seems to appear. Sometimes alpha and beta values are both higher for a modelled PAH in one soil compared to the other, sometimes they are both smaller, sometimes one is bigger and the other smaller. Also, there does not seem to be a systematic link between high parameter values and high calculated t_{ex} values. When it comes to comparing obtained models with published data in the literature, Barnier *et al.* (2014) are the only ones, to the best of our knowledge, who tested (and published the related data) the site distribution model to describe PAHs desorption kinetics in three aged-contaminated soils. Their tested soils presented total PAHs concentrations of 1670 mg kg^{-1}, 668 mg kg^{-1}, and 773 mg kg^{-1}, which is rather similar to the two experimental soils exposed in Table 15. They established the desorption kinetics of the same 15 PAHs as presented here and tested three models to describe their data (the site distribution model, the first-order two-compartment model, and the first-order three-compartment model). They ended up selecting the first-order two-compartment model to assess bioaccessibility (also using a Tenax® extraction method), but also published the fitted parameters from the unselected models. Unlike the data presented in Table 16, their obtained alpha and beta values were overall much closer in range (from 2 x 10^{-3} to 4.18 x 10^{-2} for alpha values and from 9 x 10^{-2} h to 239 h for the beta values) throughout all modelled PAHs and tested experimental soils. As a reminder, alpha values from this thesis' models range from 4.40 × 10^{-4} to 1.14 x 10^{-2} and beta values range from 2.17 × 10^{-7} h to 1.86 h. When it comes to other PAHs desorption models published in the literature, and as was previously explained in the general introduction, the most used model is the first-order two-compartment model, thus fitted parameters are different and can not be compared.

Table 15. Initial PAHs total extractable contents of the two experimental soils used in the thesis. Values are means ± confidence intervals (5%).

PAH	**Soil from Saint-Ghislain (mg kg^{-1} DW)**	**Soil from Marchienne-au-Pont (mg kg^{-1} DW)**
N	28.9 ± 1.7	20.2 ± 2.4
Ace	19.4 ± 1.2	1.0 ± 0.4
Fle	12.5 ± 1.1	5.1 ± 0.9
Phen	46.5 ± 5.5	45.5 ± 7.2
Anthr	16.0 ± 1.4	24.1 ± 3.6
∑2-3 rings	**123.2 ± 8.2**	**95.9 ± 12.2**
F	65.9 ± 7.1	139.1 ± 36.6
Pyr	45.6 ± 4.8	117.4 ± 20.5
∑4 rings	**111.5 ± 11.9**	**256.5 ± 47.9**
BaA	28.3 ± 3.6	79.2 ± 10.5
Chrys	32.4 ± 4.0	73.6 ± 8.5
BbF	23.1 ± 3.3	96.0 ± 19.4
BkF	11.8 ± 1.6	48.1 ± 5.0
BaP	18.3 ± 2.6	95.2 ± 15.6
DBahA	2.9 ± 0.1	12.1 ± 1.3
BghiP	14.1 ± 3.6	66.3 ± 25.3
IcdP	15.0 ± 2.6	94.3 ± 21.7
∑4-6 rings	**145.9 ± 20.6**	**564.8 ± 90.0**
∑all	**380.5 ± 39.8**	**917.2 ± 145.4**

The most interesting comparison that can be made between the two tested experimental soils (from Saint-Ghislain and Marchienne-au-Pont) is by analyzing the initial bioaccessible contents that were measured. As exposed in Table 15, the soil from Saint-Ghislain had a total extractable PAHs content that was about half the content in the Marchienne-au-Pont experimental soil (381 ± 39.8 mg kg^{-1}DW and 917 ± 146 mg kg^{-1}DW, respectively). Yet, its initial bioaccessible contents (exposed in Table 17) were systematically higher than the bioaccessible contents in the soil from Marchienne-au-Pont (from almost 2.6-fold for the ∑4-6 rings group to almost 14-fold for the ∑2-3 rings group). Let us keep in mind that the calculated (and used) Tenax® extraction times were different for both soils (48 h for the soil in Saint-Ghislain and 24 h for the soil in Marchienne-au-Pont). But these extraction times were calculated the same way and represent "the time for the most accessible PAH fraction to equilibrate with Tenax® beads", i.e. the time in which the slope from each model closes down to zero (Davin *et al.*, 2019 and 2020). Thus, it is reasonable to assume that the differences in the PAHs bioaccessible contents are caused by other factors, such as the ones related to ageing. Indeed, and as exposed in the general introduction, many parameters influence the fate of hydrophobic organic compounds in the soil, leading to more or less adsorption and sequestration onto and into soil particles. So those soils probably stand at different ageing stages that were influenced by the age of the pollution itself, humidity, soil's physico-chemistry, rainfalls…

These differences in total and bioaccessible contents between the two experimental soils meet the statement developed in Part 4, suggesting that the risk represented by a pollution does not only lie in its global content but also in its bioaccessible one. Indeed, the global bioaccessible content of the soil from Marchienne-au-Pont is a lot lower than in the soil from Saint-Ghislain, suggesting the pollution actually might present less of a risk than its total extractable content suggests. Of course, this bioaccessible parameter alone does not allow to make a clear statement, and it would have to be added to other soil characteristics into a risk-analysis to make a better estimation. But it certainly is a good example of the fact that bioaccessible contents could bring valuable information in contaminated land management and remediation decision-making.

Table 16. Fitted parameters of the site distribution model and t_{ex} values calculated for different PAHs in the two experimental soils used in the thesis.

PAH	**β (h)**		**α (-)**		**t_{ex} (h)**	
	Soil from Saint-Ghislain	**Soil from Marchienne-au-Pont**	**Soil from Saint-Ghislain**	**Soil from Marchienne-au-Pont**	**Soil from Saint-Ghislain**	**Soil from Marchienne-au-Pont**
N	1.54×10^{-2}	1.86×10^{0}	1.53×10^{-3}	4.41×10^{-3}	24	24
Ace	6.82×10^{-4}	7.30×10^{-2}	1.22×10^{-3}	3.72×10^{-3}	24	24
Fle	8.98×10^{-4}	1.06×10^{-1}	2.83×10^{-3}	2.05×10^{-3}	24	24
Phen	2.00×10^{-3}	4.39×10^{-2}	3.91×10^{-3}	1.33×10^{-3}	48	24
Anthr	9.30×10^{-3}	6.94×10^{-2}	1.27×10^{-2}	1.53×10^{-3}	48	24
F	1.05×10^{-2}	1.09×10^{-1}	4.61×10^{-3}	1.37×10^{-3}	48	24
Pyr	2.43×10^{-3}	4.92×10^{-3}	4.14×10^{-3}	4.77×10^{-4}	48	24
BaA	1.02×10^{-1}	8.84×10^{-2}	1.14×10^{-2}	1.62×10^{-3}	48	24
Chrys	1.24×10^{-1}	1.32×10^{-1}	1.53×10^{-2}	2.20×10^{-3}	48	24
BbF	2.78×10^{-1}	6.57×10^{-3}	1.24×10^{-2}	1.04×10^{-3}	48	24
BkF	6.03×10^{-1}	2.13×10^{-2}	1.45×10^{-2}	1.41×10^{-3}	48	24
BaP	5.54×10^{-1}	6.09×10^{-4}	1.12×10^{-2}	6.84×10^{-4}	48	24
DBahA	1.34×10^{0}	2.17×10^{-7}	1.15×10^{-2}	4.40×10^{-4}	48	24
BghiP	1.95×10^{-1}	5.93×10^{-7}	4.66×10^{-3}	5.38×10^{-4}	48	24
IcdP	5.29×10^{-1}	6.73×10^{-5}	6.88×10^{-3}	5.61×10^{-4}	48	24

Table 17. Initial PAHs bioaccessible contents of the two experimental soils used in the thesis. Values are means ± confidence intervals (5%).

PAH	**Soil from Saint-Ghislain ($\mu g\ g^{-1}$ DW)**	**Soil from Marchienne-au-Pont ($\mu g\ g^{-1}$ DW)**
∑2-3 rings	4.61 ± 0.52	0.33 ± 0.06
∑4 rings	4.42 ± 0.33	0.84 ± 0.08
∑4-6 rings	9.09 ± 0.77	3.46 ± 0.60
∑all	18.12 ± 1.57	4.62 ± 0.73

2. Impact of the different treatments on microbial health

Three amendments were tested on an aged-contaminated soil during incubation experiments in microcosms (commercial saponin from *Quillaja saponaria* Molina bark, *Medicago sativa* L. root exudates, and *Trifolium pratense* L. root exudates). CO_2 emissions and dehydrogenase activities were determined throughout the incubation and at the end of the incubation periods, respectively, as ways to rapidly evaluate the potential toxicity of the amendments and the soil global microbial activity. All the tested amendments (Sap2.5, Sap5, E_MS and E_TP), compared to control samples (C) led to similar observations in dehydrogenase activities. Whilst dehydrogenase activities were decreasing in C samples throughout the incubation, all amendments led to significant increases in dehydrogenase activities after 14 and 28 days of incubation. But in all cases, the activities at 28 days were starting to diminish compared to activites at 14 days, even though there were still statistically similar (Figure 4 and Figure 10). When it comes to CO_2 emissions (Figure 3 and Figure 9), significant increases were measured throughout the whole incubation for Sap2.5, Sap5 and E_TP samples, compared to C samples. However E_MS samples did not emit significantly more CO_2 than C samples, at any time during the 28 days of incubation, even though they did not emit less CO_2 than C samples either.

These observations suggest that none of the tested amendments showed toxicity towards the general soil microbiota. But given the observations made on PAHs residual contents (hereafter), the data also suggests that Sap2.5, Sap5 and E_TP amendments were most likely used by the soil microbiota as primary, more readily accessible, carbon sources. E_MS amendments, however, were not as degraded by the microbiota, even though their presence led to enhanced dehydrogenase activity.

3. Impact of the different treatments on the PAHs bioaccessibility and dissipation

PAHs bioaccessibility was assessed in different soils (from Saint-Ghislain in Part 1 and Part 2 and from Marchienne-au-Pont in Part 3), before and after different treatments (incubation in microcosms with commercial saponin from *Q. saponaria* Molina bark in Part 1, incubation in microcosms with *M sativa* L. or *T. pratense* L. root exudates in Part 2, and rhizoremediation with *M sativa* L. or *T. pratense* L. plants in Part 3). In these cases, bioaccessibility was assessed using a Tenax® beads extraction method. But prior to that, an extraction experiment using aqueous solutions of commercial saponin (0, 1, 2, 4 and 8 g L^{-1}) was conducted and apparent aqueous solubility was used as a proxy to assess PAHs bioaccessibility (Part 1). Even though this latter protocol was not used in the incubation and rhizoremediation experiments, it still led to a few interesting observations, as will be discussed below. PAHs dissipation was assessed in the soils from Saint-Ghislain (Part 1 and Part 2) and from Marchienne-au-Pont (Part 3), before and after the same incubation experiments and rhizoremediation trial. This was achieved by measuring the residual (or total extractable) PAHs contents through a more exhaustive extraction protocol.

Bioaccessible and residual (total extractable) contents evolved slightly differently according to the different incubation and rhizoremediation experiments.

In both incubation experiments (with commercial saponin or with plant root exudates), the simple humidification, mixing, and pre-incubation of aged-contaminated soil samples allowed for an important increase in all groups of PAHs bioaccessible contents (there was no “pre-incubation period” in the rhizoremediation trial). Afterwards, there are many similarities that appear between the bioaccessible contents’ evolution during the incubation in presence of commercial saponin, MS root exudates, and TP root exudates, with one exception. However, there are a few more dissimilarities that appear between the residual contents evolution of the samples during the incubation experiments. As a reminder, similar groups of PAHs bioaccessible contents exhibited similar patterns in presence of commercial saponin, regardless of the type of treatment they received, and the patterns were also overall similar between the PAHs groups, suggesting that commercial saponin had no effect on the PAHs bioaccessibility compared to untreated samples (Figure 5). When it comes to residual contents, the same observation was made for all groups of PAHs, as similar groups also exhibited similar patterns, no matter the received treatment (Figure 6). This also suggests a lack of effect of the commercial saponin on the PAHs dissipation. In presence of MS or TP root exudates, the same observations can be made on the bioaccessibility patterns (Figure 12). Overall, similar groups of PAHs exhibited similar patterns regardless of the type of amendment (MS or TP exudates) that the soil samples received. The only exception lies in the fact that the total (∑all) bioaccessible PAHs content decreased faster in presence of MS root exudates than in untreated control samples, and also that this amendment led to lower total bioaccessible contents than TP root exudates both after 14 and 28 days of incubation. When examining the residual contents evolution (Figure 11), different patterns were described for the different PAHs groups, according to the received treatment (C, E_MS, E_TP). Residual contents did not lower significantly in any of the samples for the heavy PAHs (∑4-6 rings). However, in E_MS samples, the ∑2-3 rings contents lowered in a slower way than in C and E_TP samples, and the ∑4 rings and ∑all groups did not significantly decrease, whereas they did in C and E_TP samples. In E_TP samples, the intermediate (∑4 rings) and global (∑all) residual contents lowered a little faster than in C samples, with similar end-points. Firstly, these patterns clearly suggest that there was a different effect on the total bioaccessible content according to the nature of the amendment (*M. sativa* or *T. pratense*). Secondly, *T. pratense* exudates did not enhance PAHs dissipation after 28 days of incubation, compared to untreated soil, whereas *M. sativa* exudates seemed to decrease PAHs global dissipation. One of the reasons might be that MS exudates significantly decreased PAHs’ bioaccessibility.

A comparison of the bioaccessible and residual contents patterns during the rhizoremediation trial (in presence of *M. sativa* L. or *T. pratense* L. plants compared to no culture, and for 3, 6 or 12 months) and the patterns during the incubation in presence of these plants' respective roots exudates was made.

As a reminder, in the rhizoremediation trial, all groups of PAHs residual contents exhibited similar patterns within each type of treatment (unlike during the incubation where patterns varied both with the type of amendment and the PAHs group). Overall, all residual PAHs contents significantly diminished throughout the whole experiment in C samples, they diminished abruptly after 3 months then remained statistically similar after 6 and 12 months in presence of *T. pratense*, and they displayed an unexpected pattern in presence of *M. sativa*. In this case, residual contents lowered after 3 and 6 months of culture, but after 12 months of culture, they were not as significantly low as after 6 months of culture (Figure 15a-Figure 18a). As for the bioaccessible PAHs contents, patterns were overall similar for each similar PAHs group, regardless of the type of culture. ∑2–3 rings and ∑4 rings bioaccessible contents increased throughout the trial, but more significantly in the absence of plants (C samples). ∑4–6 rings and ∑all contents did not significantly differ.

These observations, when confronted to the observations made during the incubation in presence of root exudates, suggest that the presence of *M. sativa* L. or *T. pratense* L., have influenced the PAHs bioaccessible and residual contents evolution compared to C samples, but possibly in different ways.

In presence of *T. pratense*, the residual contents diminished more abruptly than in unplanted samples. Indeed, after 3 months, the residual contents of all PAHs groups are significantly lower in presence of *T. pratense* than in unplanted samples, even though on the long term (12 months) all contents are statistically equivalent in TP and C samples. A similar observation was made in the incubation trial in presence of *T. pratense* exudates (reminded here-above). These observations, coupled to the fact that CO_2 emissions and dehydrogenase activities were also significantly higher in samples incubated in presence of these exudates, suggest that *T. pratense* exudates, whether they were added in a single dose (in the incubation trial), or in a continuous way (in the rhizoremediation trial), could have been used as a primary carbon source by the soil microbiota, increasing its global health and giving it a head start in the dissipation of PAHs. This tends to confirm the potential of *Trifolium pratense* L. for rhizoremediation. However, the residual contents in TP samples were similar after 3, 6, and 12 months. This could be explained by the fact that TP plants did not grow well on the aged-contaminated soil during those periods (Part 3), meaning *T. pratense* would have to be cultured in more agronomically suitable conditions in order to efficiently enhance PAHs remediation.

In presence of *M. sativa*, observations are a little more puzzling. On the one hand, the incubation trial results suggest that MS exudates negatively influenced the PAHs' dissipation by decreasing the PAHs' bioaccessible contents in a more significant way than happened in other samples. On the other hand, the rhizoremediation trial suggests that MS plants slowed the increase of the bioaccessible contents, compared to C samples, whilst still showing significant decreases in the residual contents, at least after 3 and 6 months of culture. The diminutions after 3 and 6 months, however, were not as significant as for the TP samples, neither were they more significant than in C samples, suggesting indeed that MS plants did not enhance PAHs dissipation and might even have slowed it through the slowing of the bioaccessibility increase. The real surprise came with the MS samples residual contents after 12 months, which were not as low as after 6 months. Several explanations have already been explored in Part 3 and will not be reminded here, but many interesting information could be brought from repeating the trial and, among others, determining whether PAHs could be pumped vertically by plants, or even be adsorbed and desorbed from plants roots, according to weather or even to the plants growth cycle.

Going back to the observations that were made on PAHs contents extracted by aqueous solutions of commercial saponin, a link could be made between those data and some of the observations that were just summarized. A suggestion was made that when the commercial saponin's concentration was too high, saponins acted as a secluding agent rather than a solubilizing agent and prevented PAHs from being extracted in aqueous solutions. Other searchers have already mentioned that surfactants sometimes act as secluding agents and have highlighted several possible mechanisms (Haigh, 1996; Zhou *et al.,* 2011). Surfactants can either seclude hydrophobic compounds into hemimicelles or micelles, then bind to soil particles, or not. In hemimicelles, a layer of surfactants binds to soil particles, decreasing the apparent solubility of a hydrophobic compound and increasing its sorption to solids. In micelles, surfactants can also bind to soil particles, which will result in similar effects; or they can remain in solution, which will result in the increase of the apparent solubility of a hydrophobic compound. The latter option seems interesting from a "washing technology" point-of-view, as it would allow to extract pollutants from a soil. But from a bioremediation perspective, the fact that a hydrophobic compound is being solubilised into a micelle does not mean it will be more degraded by the soil microorganisms. If the surfactants are not degradable by the microorganisms, then they can act as secluding agents too, even if they have increased the apparent solubility of a compound. This could actually explain the fact that bioaccessible contents did not increase, or did not increase as fast, as in controls when confronted to plant-based treatment, especially in the case of experiments involving *Medicago sativa* L. or its exudates. The whole thesis was based on the postulate that because *M. sativa* L. and *T. pratense* L. were reported to produce saponins in their roots (Vincken *et al.,* 2007), they could act as bioaccessibility enhancers and thus increase PAHs dissipation in aged-contaminated soil. But it is also possible that the opposite effect happened and that one or several components of the plants roots or root exudates interacted with the PAHs in a negative way (from a biodegradation point-of-view). The interaction could be seclusion by surface-active compounds, as was previously suggested, but it could also be plain sorption to other constituents of the plants (such as the root cells' lipidic membranes) (Ouvrard *et al.,* 2014). Finally, the fact that surface-active compounds could actually increase a hydrophobic pollutant's apparent aqueous solubility whilst still secluding it from degrading agents is one of the reasons why it is more interesting to investigate a treatment's effect on a pollutant's bioaccessibility through a biomimetic method (as was achieved with the Tenax® beads) than through an aqueous extraction trial. Of course, the bioaccessibility assessment alone can not account for the complex processes that take place in the soil. For example, whether the Tenax® “mimics” a surfactant’s degradation by the microbiota remains uncertain. It is possible that the resin actually sorbs both freely dissolved pollutants and pollutants that are dissolved into surfactants micelles, but it would have to be verified. In the meantime, just like bioaccessibility is a complementary information to total extractable contents, it is still important to evaluate a pollutant’s dissipation along with its bioaccessibility in order to assess whether a treatment enhances bioremediation or not.

4. Conclusions, recommendations and perspectives

The remediation of polluted soils is a complex matter that remains unfamiliar to the general public and tends to be underestimated in political choices. As naïve as it may sound, the most obvious solution to remediate soil, and environmental, pollution is to prevent contaminations from happening in the first place. Several legislations have been implemented to prevent pollutions and make the polluters accountable for their actions, as a first step towards sustainable environmental management. But Wallonia carries a heavy environmental debt inherited from a glorious industrial past, and there is still research, and decisions, to make to develop remediation treatments.

This thesis aimed at the investigation and development of more eco-friendly, cheaper biological remediation techniques that would be best suited for polluted brownfields that are less of a priority. It was also based on the assumption, reported by several searchers, that the presence of vascular plants could lower PAHs contents in contaminated soil. Because they are at the centre of the majority of soil remediation challenges, aged-contaminated soils from brownfields were chosen to host the experiments.

The thesis’ principal hypothesis was that *Medicago sativa* L. and/or *Trifolium pratense* L., through their root exudates, could influence the PAHs bioaccessibility and thus enhance their remediation. The results clearly suggest that from a remediation point-of-vue, none of the exposed treatments have significantly enhanced PAHs dissipation compared to unamended or unplanted soil. This does not mean that this field of research should be abandoned because the few experiments that were led, and their different outcomes, have probably asked more questions than they answered. Indeed, the discussion above was based on the experiments made on two aged-contaminated soils, in presence of two plants (and/or their exudates), and none of the assumptions and hypotheses that were formulated can be turned into general statements.

Clearly, there is still a lot of characterization and experimentation that must be done to bring more information to the debate. Both tested plants, and their root exudates, could be further investigated in their long-term effects, or even as temporary remediation solutions. For instance, *M. sativa* plants could be investigated as phytopumps that would slow the vertical migration of pollutants in soil, hence helping the preservation of groundwater. Also, if plant root exudates eventually turned out effective in reducing bioaccessibility, they could be used (either as amendment or *in situ*) to temporarily help reduce a soil's toxicity. As for *T. pratense*, it showed potential for a more traditional rhizoremediation application, and it is worth exploring on the long-term whether this species could enhance bioremediation, even if it simply is by enhancing a soil's microbial health. Given the fragility that this species exhibited towards more extreme soil physico-chemical parameters though, it would probably require amendments to increase plants growth. More generally, and since the composition of plants root exudates can also vary depending on the season or the growth conditions, more extensive testing of those plants or others should be conducted on a large variety of aged-contaminated soils, for several years (or at least a period somewhat longer than the length of a thesis), in order to properly assess whether they are actually bioremediation enhancers, or even could be used in other ways.

Besides, a few more elements could be investigated, such as the fate of PAHs on or inside the plants, but also the characterization of the resulting PAHs' metabolites. Indeed, it is important to keep in mind that the objective of soil remediation is to lower toxicity and risk towards the environment at large. Bringing pollution contents under quality guidelines levels is nonsense if bigger threats are introduced into the environment in the process.

The observations reported in this work have shown that not all plants are capable of enhancing PAHs remediation. But plants also might not be capable of enhancing PAHs remediation in any type of soil. Soils are a tremendous source of variation and what was observed in these experiments might not be valid anymore with different soils. Indeed, the tested experimental soils were both heavily polluted. They both presented high PAHs contents, as well as co-contaminants (trace elements, petroleum hydrocarbons…) Figure 20 presents the expected concentrations of the 16 US-EPA PAHs in the Walloon soils. This map results from the sampling of several areas, followed by modelisation to extrapolate to the whole territory. It represents the levels of PAHs (∑tot) contamination that are to be expected based on the characterization of different soil types that were exposed to different levels of atmospheric diffuse pollution (Leclercq, 2015). The estimated concentrations range up to about 17 mg kg^{-1} DW, which can be found in areas known for their heavy industrial past (where the experimental soils of this thesis originate from). Both experimental soils presented levels of PAHs contaminations that where higher than most threshold (VS) values for an industrial use of soils in the Walloon legislation (both the former version from 2008 and the revised one from 2018). The values encountered in the experimental soils are 20 to 50-fold superior to the concentrations encountered in Figure 20. On the one hand, it is important to keep in mind that such heavy pollution did not result from diffuse pollution but rather from local, more concentrated activities (e.g. spills), meaning they do not constitute exceptions in the panel of polluted soils that can be present in the Walloon region. On the other hand, this map shows that not all soils are as heavily polluted as the experimental ones, and that many areas that are less polluted, but still require remediation, might present with PAHs concentrations more suited for biological remediation techniques. Indeed, soil remediation is a complex matter, and not every type of remediation treatment is suited for any type of polluted soil nor any level of contamination. The soil from Saint-Ghislain and its total PAHs content of about 139 mg kg^{-1}DW might have been best suited for a more agressive, even though more expensive and destructive remediation treatment such as a chemical oxidation; and the soil from Marchienne-au-Pont, which was much more contaminated with about 917 mg kg^{-1}DW of PAHs, might even have required an encapsulation treatment, in order to contain a much more important pollution.

Besides, and since most brownfields present with multiple contaminations, it would be of great interest to study the effect of biological treatments (or any other type of treatment) on several types of contaminants at a time, because this too would be more representative of the actual remediation challenges.

As the matter of legislation was just mentioned, it is important to emphasize a few important changes that took place during the course of this thesis in the Walloon legislation (Table 18). The original legislation (Décret relatif à la gestion des sols, 2008), to which the soil from Saint-Ghislain was confronted in Part 1, was much more complex, but also less permissive than is the revised version (Décret relatif à la gestion des sols, 2018). The Walloon legislation seperates historical pollution (arbitrarily set to have happened before April 30th 2007) from new pollution (which thus happened after this date). The original legislation presented three sets of values for each pollutant and for several types of soil occupation (industrial, commercial, residential, agricultural or natural). The intervention value (VI) was the value over which soils were to be cleaned-up, the threshold value (VS) was the value above which a risk-assessment (in case of a historical pollution), and a mandatory clean-up (in case of a new pollution) were to be implemented, and the reference value (VR) was the ideal value to reach for the remediation of historical pollutions, and it was the mandatory value to reach for the remediation of a new pollution. Rather than completely cleaning up a pollution, which could both turn out expensive and unnecessary, as was discussed previously, the philosophy for historical pollution was thus to eliminate the risks and threats towards the environment. Therefore, remediation end-points were determined for each case scenario based on risk-assessments. Also, it is important to keep in mind that the VS, for each pollutant and each type of soil occupation, were the lowest of three partial VS calculated for human health, ecosystems, and groundwater through ecotoxicity analyses (SPAQuE, 2010). The new legislation is simpler than the original one because it now only contains updated VS. But as mentioned previously, it is also more permissive. The legislation was very openly adapted based on the arguments that cleaning up new pollutions back to VR was expensive. Therefore, updated VS have overall been raised compared to the original values, especially for industrial and commercial soil occupation types. Let us also specify that updated VS for commercial and industrial uses do not account for risks towards the ecosystems anymore, suggesting that soils hosting such activities have no, or less important, ecosystems. So, whilst the general management of historical pollution has not changed in the new legislation, besides the fact that VS are now higher, the management of new pollutions is very different. Indeed, not only are VS higher, rendering clean-up obligations less frequent, but remediation end-points are now set to 80% of the updated VS, whereas before the end-points were the VR (which were much lower). This inevitably raises questions regarding the way environmental pollution is being managed. As mentioned previously, remediation techniques can indeed be very expensive, especially applied to aged, historical pollution. The re-evaluation of legislation and remediation end-points is in adequation with what has been advised in Part 4 of this thesis. However, other parameters such as the pollutants bioaccessibility should be included in the process. But this change of legislation concerning new pollution might be sending the wrong message, especially at a time when environmental awareness should be rising. There are other legislations that exist to prevent pollution, and this one might make the polluters less accountable for their actions than they ought to be.

Finally, bioaccessibility was mainly developed in the literature as a risk-assessment tool. This thesis approached bioaccessibility in an original way by using it as a tool to study and investigate PAHs remediation in presence of plants or their material. In the meantime, similar approaches have started to be shared in the literature. This should be encouraged, and generalized in the same way that the assessment of total extractable contents is classically used to characterize a pollution in soil management, for bioaccessibility definitely is an important key to developing sustainable and effective soil remediation strategies. But furthermore, a systematic use of PAHs (or pollutants in general) bioaccessibility in the characterization of brownfields might lead to different approaches in their management. For instance, such measurement could suggest that a brownfield does not present a risk towards the environment, if the land is allocated to specific and controlled uses such as the production of biomass (e.g. the growth of willow trees) or the culture of non-edible plants (e.g. textile hemp). This would mean implementing management strategies that are not necessarily based on pollution remediation.

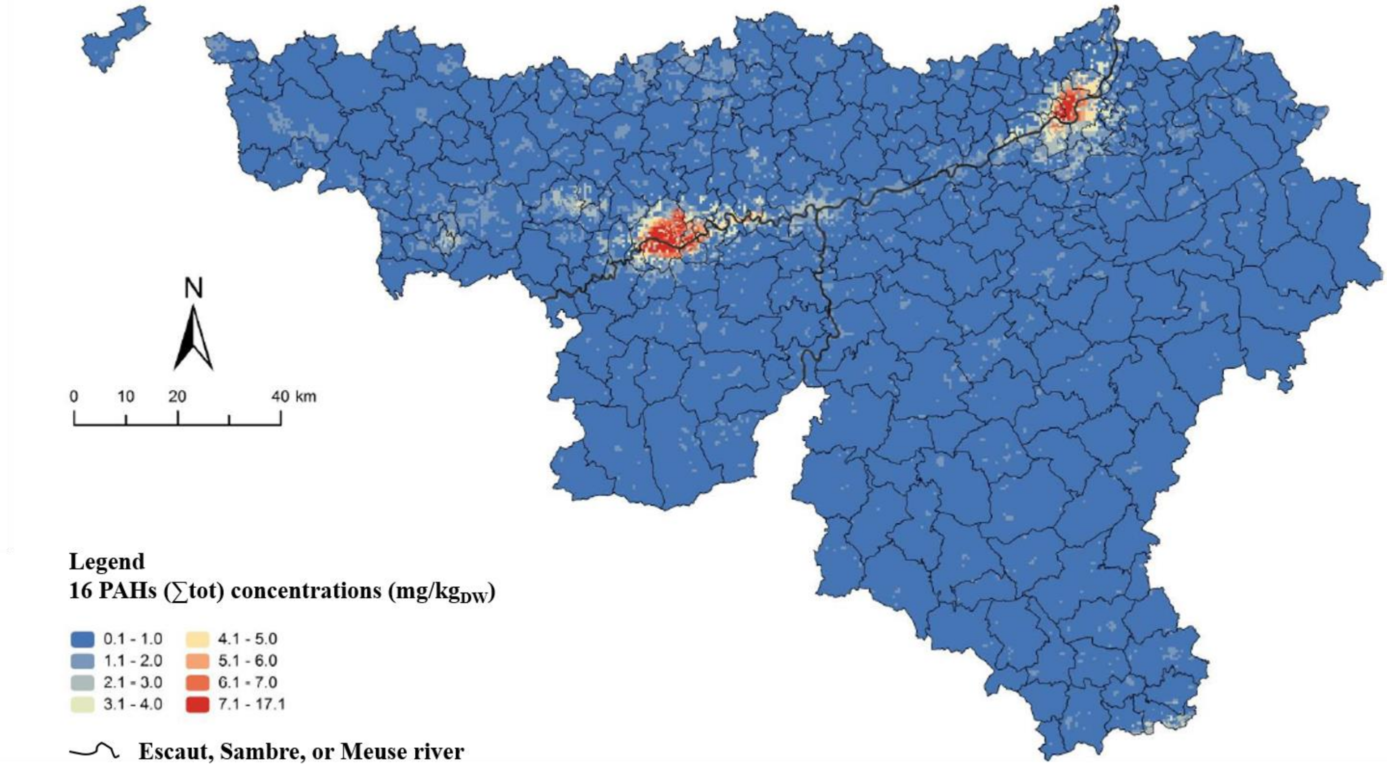


Figure 20. Expected concentrations of the 16 US-EPA PAHs (∑tot) in the soils of the Walloon region (Belgium) (adapted from Leclercq, 2015).

Table 18. Comparaison of the PAHs norms for soils of the Wallon region (Belgium) in the former (2008) and revised (2018) legislations. **VR** (Reference Value) is the ideal value to reach for the remediation of historical pollutions, and mandatory value to reach for the remediation of a new pollution in the 2008 legislation; **VS** (Threshold value) is the value above which a risk-assessment (in case of a historical pollution), and a mandatory clean-up (in case of a new pollution) are to be implemented in both legislations; **VI** (Intervention value) is value over which soils were to be cleaned-up in the 2008 legislation. **Values in bold** were raised in the 2018 legislation compared to the 2008 legislation.

		Soil (mg/kg$_{DW}$)				
Occupation		**natural**	**agricultural**	**residential**	**recreational or commercial**	**industrial**
Naphthalene (N)	VR	0.1	0.1	0.1	0.1	0.1
	VS (2008)	1.1	0.7	1.7	1.7	2.5
	VS (2018)	**2.5**	**2.5**	**2.5**	**2.5**	**6.3**
	VI	4	2.5	9	9	25
Acenaphtylene (A)	VR	0.01	0.01	0.01	0.01	0.01
	VS (2008)	0.3	0.3	0.8	8	43
	VS (2018)	**4.8**	**4.8**	**6.3**	8	43
	VI	3	3	8	78	410
Acenaphtene (Ace)	VR	0.01	0.01	0.01	0.01	0.01
	VS (2008)	2.6	1.6	3.9	3.9	6
	VS (2018)	2	**2**	**4**	**4**	6
	VI	9	6	19	19	56
Fluorene (Fle)	VR	0.01	0.01	0.01	0.01	0.01
	VS (2008)	4	2	9	9	16
	VS (2018)	**5.9**	**5.9**	9	9	16
	VI	26	16	46	46	163
Phenanthrene (Phen)	VR	0.1	0.1	0.1	0.1	0.1
	VS (2008)	9	6	12	12	16
	VS (2018)	7.6	**7.6**	**13**	**13**	**25**
	VI	27	16	60	60	164
Anthracene (Anthr)	VR	0.01	0.01	0.01	0.01	0.01
	VS (2008)	0.3	0.2	0.7	0.7	1.3
	VS (2018)	**2.8**	**2.8**	**2.8**	**2.8**	**6.9**
	VI	2.2	1.3	3.7	3.7	13.3

(continued)

Occupation		**Soil (mg/kg$_{DW}$)**				
		natural	**agricultural**	**residential**	**recreational or commercial**	**industrial**
Fluoranthene (F)	VR	0.01	0.01	0.01	0.01	0.01
	VS (2008)	8	5	23	23	47
	VS (2018)	6	**6**	11.6	23	47
	VI	77	48	126	126	475
Pyrene (Pyr)	VR	0.01	0.01	0.01	0.01	0.01
	VS (2008)	1.4	0.9	3.6	3.6	6.4
	VS (2018)	**6.7**	**6.7**	**13**	**15.4**	**28.6**
	VI	10	6	18	18	64
Benzo[a]anthracene (BaA)	VR	0.01	0.01	0.01	0.01	0.01
	VS (2008)	0.8	0.5	1	1	1.5
	VS (2018)	**9.5**	**9.5**	**9.5**	**9.5**	**17.3**
	VI	2.5	1.5	5	5	15
Chrysene (Chrys)	VR	0.01	0.01	0.01	0.01	0.01
	VS (2008)	5	3	5	5	6
	VS (2018)	1.1	1.1	2.3	**9.7**	**17.6**
	VI	10	6	25	25	60
Benzo[b]fluoranthene (BbF)	VR	0.01	0.01	0.01	0.01	0.01
	VS (2008)	0.7	0.4	0.3	0.9	1.3
	VS (2018)	**1.7**	**1.7**	**3.3**	**11**	**21**
	VI	2	1.5	4	4	13
Benzo[k]fluoranthene (BkF)	VR	0.01	0.01	0.01	0.01	0.01
	VS (2008)	2.5	1.6	1.3	3.1	4.7
	VS (2018)	1	1	**2**	**5.3**	**9.3**
	VI	7.6	4.7	12.8	15.5	47
Benzo[a]pyrene (BaP)	VR	0.01	0.01	0.01	0.01	0.01
	VS (2008)	0.2	0.2	0.5	0.9	1.3
	VS (2018)	**0.87**	**0.87**	**3.6**	**9.5**	**14.4**
	VI	2.2	1.3	4.5	4.5	13

(continued)

Occupation		**Soil (mg/kg$_{DW}$)**				
		natural	**agricultural**	**residential**	**recreational or commercial**	**industrial**
Dibenzo[ah]anthracene (DBahA)	VR	0.01	0.01	0.01	0.01	0.01
	VS (2008)	0.8	0.1	0.6	1	1.4
	VS (2018)	**0.81**	**0.81**	**1.8**	**1.8**	**3.2**
	VI	2.3	0.7	5	5	14
Benzo[g,h,i]perylene (BghiP)	VR	0.01	0.01	0.01	0.01	0.01
	VS (2008)	2.5	1.5	3	3	5
	VS (2018)	0.8	0.8	1.5	**6.8**	**11.1**
	VI	7	5	15	15	46
Indeno[1,2,3-c,d]pyrene (IcdP)	VR	0.01	0.01	0.01	0.01	0.01
	VS (2008)	1	0.6	0.2	1.2	1.5
	VS (2018)	**4.5**	**4.5**	**7**	**7**	**12**
	VI	2.5	1.5	2.5	6	15

5. References

Barnier, C., Ouvrard, S., Robin, C., Morel, J.L., 2014. Desorption kinetics of PAHs from aged industrial soils for availability assessment. *Sci Total Environ.,* 470-471:639-645. https://doi.org/10.1016/j.scitotenv.2013.10.032

Davin, M., Starren, A., Marit, E., Lefébure, K., Fauconnier, M-L., Colinet, G., 2019. Investigating the Effect of *Medicago sativa* L. and *Trifolium pratense* L. Root Exudates on PAHs Bioremediation in an Aged-Contaminated Soil. *Water Air Soil Pollut.,* 230-296. https://doi.org/10.1007/s11270-019-4341-4

Davin, M., Renard, E., Lefébure, K., Colinet, G., Fauconnier, M.-L., 2020. Temporal evolution of PAHs bioaccessibility in an aged-contaminated soil during the growth of two *Fabaceae*. *Int. J. Environ. Res. Public Health,* 17(11), 4016. https://doi.org/10.3390/ijerph17114016

Décret relatif à la gestion des sols, 2008 (Moniteur Belge du 18/02/2009, p. 14852. Add.: Moniteur Belge du 06/03/2009, p. 19935)

Décret relatif à la gestion des sols, 2018 (Moniteur Belge du 22/03/2018, p. 28679. Add : Moniteur Belge du 20/02/2019, p. 17489)

Haigh, S., 1996. A review of the interaction of surfactants with organic contaminants in soil. *Sci. Total Environ.*, 185 (1-3), 161-170.

Leclercq, J., 2015. Pollusol 2, (2009-2015) - Rapport de synthèse - Investigations des zones de pollution atmosphérique de proximité en Wallonie. SPAQuE, service de l'évaluation des risques.

Ouvrard, S., Leglize, P., & Morel, J.L., 2014. PAH Phytoremediation: Rhizodegradation or Rhizoattenuation? *Int J Phytoremediation,* 16(1): 46-61. https://doi.org/10.1080/15226514.2012.759527

SPAQuE, 2010. Guide pour l'évaluation des risques, santé humaine – eaux souterraines – écosystèmes. Cahier de bonnes pratiques n° 5, CBP-5-1.1-1001, 250 p.

Vincken, J.P., Heng, L., de Groot, A., Gruppen, H., 2007. Saponins, classification and occurrence in the plant kingdom. *Phytochemistry*. 68:275–297.

Zhou, W., Yang, J., Lou, L., Zhu, L., 2011. Solubilization properties of polycyclic aromatic hydrocarbons by saponin, a plant-derived biosurfactant. *Environ. Pollut.,* 159:1198-1204. https://doi.org/10.1016/j.envpol.2011.02.001